BIOCHEMISTRY

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To:

Our parents, who encouraged us,
Our teachers, who enabled us, and
Our children, who put up with us.

Cover Art: One of a series of color studies of horse heart cytochrome *c* designed to show the influence of amino acid side chains on the protein's three-dimensional folding pattern. We have selected this study to symbolize the discipline of biochemistry: Both are beautiful but still in process and hence have numerous "rough edges." Drawing by Irving Geis in collaboration with Richard E. Dickerson.

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Library of Congress Cataloging in Publication Data:

Voet, Donald.
Biochemistry / by Donald Voet and Judith G. Voet.
p. cm.
Includes bibliographical references.
ISBN 0-471-61769-5
1. Biochemistry. I. Voet, Judith G. II. Title.
QP514.2.V64 1990 89-16727
574.19'2—dc20 CIP

Printed in the United States of America

10 9 8 7 6 5 4 3 2

Chapter Summary

Nucleic acids are linear polymers of nucleotides containing either ribose residues in RNA or deoxyribose residues in DNA that are linked by 3′ → 5′ phosphodiester bonds. In double helical DNAs and RNAs, the base compositions obey Chargaff's rules: A = T and G = C. RNA, but not DNA, is susceptible to base-catalyzed hydrolysis.

B-DNA consists of a right-handed double helix of antiparallel sugar–phosphate chains with ~ 10 bp per turn of 34 Å and with the bases all perpendicular to the helix axis. Bases on opposite strands hydrogen bond in a geometrically complementary manner to form A·T and G·C Watson–Crick base pairs. DNA replicates in a semiconservative manner as has been demonstrated by the Meselson–Stahl experiment. At low humidity, B-DNA undergoes a reversible transformation to a wider, flatter right-handed double helix known as A-DNA. Z-DNA, which is formed at high salt concentrations by polynucleotides of alternating purine and pyrimidine base sequences, is a left-handed double helix. Double-helical RNA and RNA·DNA hybrids have A-DNA-like structures. DNA occurs in nature as molecules of enormous lengths which, because they are also quite stiff, are easily mechanically cleaved by laboratory manipulations.

When heated past its melting temperature, T_m, DNA denatures and undergoes strand separation. This process may be monitored by the hyperchromism of the DNA's UV spectrum. The orientations about the glycosidic bond and the various torsion angles in the sugar–phosphate chain are sterically constrained in nucleic acids. Likewise, only a few of the possible sugar pucker conformations are commonly observed. Watson–Crick base pairing is both geometrically and electronically complementary. Yet, hydrogen bonding interactions do not significantly stabilize nucleic acid structures. Rather, they are largely stabilized by hydrophobic interactions. Nevertheless, the hydrophobic forces in nucleic acids are qualitatively different in character from those that stabilize proteins. Electrostatic interactions between charged phosphate groups are also important structural determinants of nucleic acids.

Nucleic acids are fractionated by many of the techniques that are used to separate proteins. Hydroxyapatite chromatography separates single-stranded from double-stranded DNA. Polyacrylamide or agarose gel electrophoresis separates DNA largely on the basis of size. Very large DNAs can be separated by pulsed-field gel electrophoresis on agarose gels. Specific base sequences may be detected in DNA with the Southern transfer technique and in RNA by the similar northern transfer technique. DNA may be fractionated according to base composition by CsCl density gradient ultracentrifugation. Different species of RNA are separated by rate-zonal ultracentrifugation through a sucrose gradient.

The linking number of a covalently closed circular DNA is topologically invariant. Consequently, any change in the twist of a circular duplex must be balanced by an equal and opposite change in its writhing number, which indicates its degree of supercoiling. Supercoiling can be induced by intercalation agents. The gel electrophoretic mobility of DNA increases with its degree of superhelicity. Naturally occurring DNAs are all negatively supercoiled and must be so in order to participate in DNA replication, RNA transcription, and genetic recombination. Type I topoisomerases (nicking–closing enzymes) relax negatively supercoiled DNAs, one supertwist at a time, by creating a single-strand break, passing a single-strand loop through the gap, and resealing it. Type II topoisomerases (gyrases) generate negative supertwists at the expense of ATP hydrolysis. They do so, two supertwists at a time, by making a double-strand scisson in the DNA, passing the duplex through the break, and resealing it.

Nucleic acids may be sequenced by the same basic strategy used to sequence proteins. Defined DNA fragments are generated by Type II restriction endonucleases, which cleave DNA at specific and usually palindromic sequences of four to six bases. Restriction maps provide easily located physical reference points on a DNA molecule. In the chemical cleavage method of DNA sequencing, a defined fragment of DNA is ^{32}P-labeled at one end and subjected to a chemical cleavage process that randomly cleaves it after a particular type of base. The electrophoresis of the four differently cleaved DNA samples in parallel lanes of a sequencing gel resolves fragments that differ in size by one nucleotide. The base sequence of the DNA can be directly read from an autoradiogram of the gel. In the chain-terminator method, the DNA to be sequenced is replicated by DNA polymerase I in the presence of a [α-^{32}P]-labeled deoxynucleoside triphosphate and a small amount of the dideoxy analog of one of the nucleoside triphosphates. This results in a series of ^{32}P-labeled chains that are terminated after the various positions occupied by the corresponding base. An autoradiograph of the sequencing gel containing the four sets of fragments, each terminated after a different type of base, indicates the DNA's base sequence. RNA may be sequenced by determining the sequence of its corresponding cDNA or by directly sequencing it by a variation of the chemical cleavage method.

Oligonucleotides are indispensible to recombinant DNA technology; they are used to identify normal and mutated genes and to specifically alter genes through site-directed mutagenesis. Oligonucleotides of defined sequence are efficiently synthesized by the phosphite-triester method, a cyclic, nonaqueous, solid phase process that has been automated.

A DNA fragment may be produced in large quantities by inserting it, using recombinant DNA techniques, into a suitable cloning vector. These may be genetically engineered plasmids, bacteriophages, cosmids, or yeast artificial chromosomes (YACs). The DNA to be cloned is usually obtained as a restriction fragment so that it can be specifically ligated into a corresponding restriction cut in the cloning vector. Gene splicing may also occur through the generation of complementary homopolymer tails on the DNA fragment and the cloning vector or through the use of synthetic palindromic linkers containing restriction sequences. Introduction of a recombinant cloning vector into a suitable host organism permits the foreign DNA segment to be produced in nearly unlimited quantities. A particular gene may be isolated through the screening of a genomic library of the organism producing the gene. Genetic engineering techniques may also be used to produce otherwise scarce or specifically altered proteins in large quantities.

NCBI

Nucleotide Protein *nucleotide–nucleotide* BLAST Translations Retrieve results for an RID

Search
```
aguuuggcgaccaugguggaucagaaccguuucgggugaagccauggucugaaggggaug
acgucccuucuggcucauccacaaaaaccgucucgggugggugaggaguccuggcugugu
gggaagcagucaguauaauucccgucgugugguggugacgccucacgacguauuuguccgc
ugugcagagcguaguaccaagggcugcaccccgguuuuuguuccaagcggagggcaaccc
```

Set subsequence From: To:

Choose database nr

Now: BLAST! or Reset query Reset all

Options for advanced blasting

Limit by entrez query or select from: All organisms

Choose filter ☑ Low complexity ☐ Human repeats ☐ Mask for lookup table only ☐ Mask lower case

Expect 10

Word Size 11

Other advanced

Format

Show ☑ Graphical Overview ☑ Linkout ☑ Sequence Retrieval ☑ NCBI-gi Alignment in HTML

Use new formatter ☐ Masking Character Default(X for protein, n for nucleotide) Masking Color Black

Number of: Descriptions 100 Alignments 50

Alignment view Pairwise

Limit results by entrez query [ ] or select from: [All organisms]

Expect value range: [ ] [ ]

Layout: [Two Windows] Formatting options on page with results: [None]

Autoformat [Semi-auto]

BLAST! or Reset all

Get the URL with preset values ? Get URL

NCBI results of BLAST

BLASTN 2.2.9 [May-01-2004]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1097593839-30310-89375910930.BLASTQ4

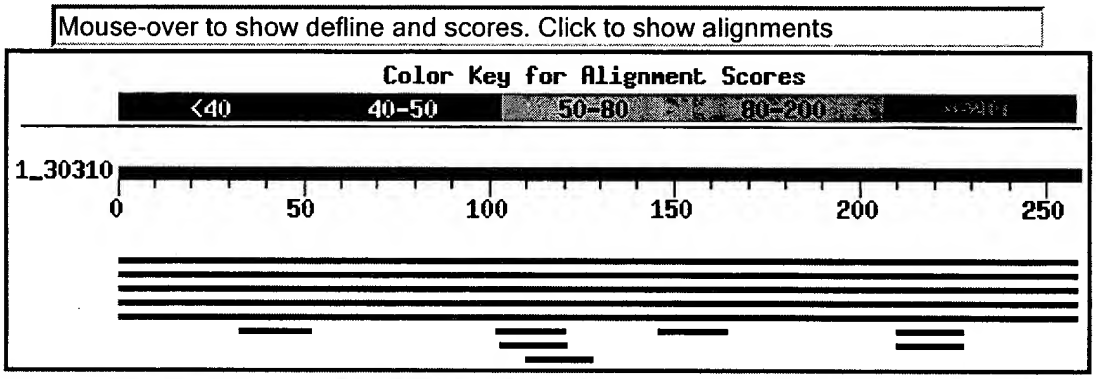
Query=
(259 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
2,635,387 sequences; 11,972,756,915 total letters

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Taxonomy reports

Distribution of 12 Blast Hits on the Query Sequence



Sequences producing significant alignments:	Score (bits)	E Value
gi|33090377|gb|AY243572.1| Synthetic construct polyprotein ...	513	e-143
gi|6014504|gb|AF179612.1|AF179612 Hepatitis GB virus B poly...	513	e-143
gi|6018427|emb|Y18973.1|HGB18973 Hepatitis GB virus B parti...	505	e-140
gi|13162187|emb|AJ277947.1|HGB277947 Hepatitis GB virus B g...	505	e-140
gi|21727885|emb|AJ428955.1|GVI428955 Hepatitis GB virus B s...	505	e-140
gi|4760437|gb|AC006316.2| Homo sapiens PAC clone RP4-672011...	40	2.3
gi|29373191|gb|AC126280.4| Mus musculus BAC clone RP23-10P2...	38	9.3
gi|28912999|gb|AC131009.7| Homo sapiens 12 BAC RP11-417L19 ...	38	9.3
gi|22094311|gb|AC093435.3| Drosophila melanogaster 3L BAC R...	38	9.3

gi|37537376|dbj|BS000109.1|	Pan troglodytes chromosome 22 c...	38	9.3
gi|28380546|gb|AE003550.4|	Drosophila melanogaster chromoso...	38	9.3
gi|8217498|emb|AL137076.6|	Human DNA sequence from clone RP...	38	9.3

Alignments

Get selected sequences | Select all | Deselect all

☐ >gi|33090377|gb|AY243572.1| Synthetic construct polyprotein gene, complete cds
Length = 9399

Score = 513 bits (259), Expect = e-143
Identities = 259/259 (100%)
Strand = Plus / Plus

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Query: 1    agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9141 agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 9200


Query: 61   acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9201 acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 9260


Query: 121  gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtatttgtccgc 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9261 gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtatttgtccgc 9320


Query: 181  tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9321 tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 9380


Query: 241  ccgcttggaattaaaaact 259
             |||||||||||||||||
Sbjct: 9381 ccgcttggaattaaaaact 9399
```

☐ >gi|6014504|gb|AF179612.1|AF179612 G Hepatitis GB virus B polyprotein gene, comp
Length = 9399

Score = 513 bits (259), Expect = e-143
Identities = 259/259 (100%)
Strand = Plus / Plus

```
Query: 1    agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9141 agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 9200


Query: 61   acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 120
```

```
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9201 acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 9260


Query: 121  gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtatttgtccgc 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9261 gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtatttgtccgc 9320


Query: 181  tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9321 tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 9380


Query: 241  ccgcttggaattaaaaact 259
            |||||||||||||||||
Sbjct: 9381 ccgcttggaattaaaaact 9399
```

☐ >gi|6018427|emb|Y18973.1|HGB18973 Hepatitis GB virus B partial 3'UTR region
Length = 357

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 Score =  505 bits (255), Expect = e-140
 Identities = 258/259 (99%)
 Strand = Plus / Plus


Query: 1    agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 99   agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 158


Query: 61   acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 159  acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 218


Query: 121  gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtatttgtccgc 180
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct: 219  gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtacttgtccgc 278


Query: 181  tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 279  tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 338


Query: 241  ccgcttggaattaaaaact 259
            |||||||||||||||||
Sbjct: 339  ccgcttggaattaaaaact 357
```

☐ >gi|13162187|emb|AJ277947.1|HGB277947 Hepatitis GB virus B genomic RNA
Length = 9397

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 Score =  505 bits (255), Expect = e-140
 Identities = 258/259 (99%)
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Strand = Plus / Plus

```
Query: 1    agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9139 agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 9198

Query: 61   acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9199 acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 9258

Query: 121  gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtatttgtccgc 180
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct: 9259 gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtacttgtccgc 9318

Query: 181  tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 9319 tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 9378

Query: 241  ccgcttggaattaaaaact 259
            |||||||||||||||||
Sbjct: 9379 ccgcttggaattaaaaact 9397
```

☐ >gi|21727885|emb|AJ428955.1|GVI428955 Hepatitis GB virus B subgenomic replicon r
Length = 8027

Score = 505 bits (255), Expect = e-140
Identities = 258/259 (99%)
Strand = Plus / Plus

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7769 agtttggcgaccatggtggatcagaaccgtttcgggtgaagccatggtctgaaggggatg 7828

Query: 61   acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7829 acgtcccttctggctcatccacaaaaaccgtctcgggtgggtgaggagtcctggctgtgt 7888

Query: 121  gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtatttgtccgc 180
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct: 7889 gggaagcagtcagtataattcccgtcgtgtgtggtgacgcctcacgacgtacttgtccgc 7948

Query: 181  tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7949 tgtgcagagcgtagtaccaagggctgcaccccggtttttgttccaagcggagggcaaccc 8008

Query: 241  ccgcttggaattaaaaact 259
            |||||||||||||||||
Sbjct: 8009 ccgcttggaattaaaaact 8027
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☐ >gi|4760437|gb|AC006316.2| Homo sapiens PAC clone RP4-672O11 from 7, complete
Length = 143369

Score = 40.1 bits (20), Expect = 2.3
Identities = 20/20 (100%)
Strand = Plus / Plus

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Query: 34     gggtgaagccatggtctgaa 53
              ||||||||||||||||||||
Sbjct: 134652 gggtgaagccatggtctgaa 134671
```

☐ >gi|29373191|gb|AC126280.4| Mus musculus BAC clone RP23-10P23 from 16, complet
Length = 211237

Score = 38.2 bits (19), Expect = 9.3
Identities = 19/19 (100%)
Strand = Plus / Plus

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Query: 104    aggagtcctggctgtgtgg 122
              ||||||||||||||||||
Sbjct: 40512 aggagtcctggctgtgtgg 40530
```

☐ >gi|28912999|gb|AC131009.7| Homo sapiens 12 BAC RP11-417L19 (Roswell Park Canc
BAC Library) complete sequence
Length = 170327

Score = 38.2 bits (19), Expect = 9.3
Identities = 19/19 (100%)
Strand = Plus / Minus

```
Query: 147    gtgtgtggtgacgcctcac 165
              ||||||||||||||||||
Sbjct: 24456 gtgtgtggtgacgcctcac 24438
```

☐ >gi|22094311|gb|AC093435.3| Drosophila melanogaster 3L BAC RP98-7A5 (Roswell I
Drosophila BAC Library) complete sequence
Length = 171689

Score = 38.2 bits (19), Expect = 9.3
Identities = 19/19 (100%)
Strand = Plus / Minus

```
Query: 211    ccggtttttgttccaagcg 229
              ||||||||||||||||||
Sbjct: 96017 ccggtttttgttccaagcg 95999
```

☐ >gi|37537376|dbj|BS000109.1| Pan troglodytes chromosome 22 clone:RP43-150L08, sequences
Length = 157620

Score = 38.2 bits (19), Expect = 9.3
Identities = 19/19 (100%)
Strand = Plus / Plus

```
Query: 103    gaggagtcctggctgtgtg 121
               ||||||||||||||||||
Sbjct: 135186 gaggagtcctggctgtgtg 135204
```

☐ >gi|28380546|gb|AE003550.4| Drosophila melanogaster chromosome 3L, section 35 sequence
Length = 304975

Score = 38.2 bits (19), Expect = 9.3
Identities = 19/19 (100%)
Strand = Plus / Plus

```
Query: 211    ccggtttttgttccaagcg 229
               ||||||||||||||||||
Sbjct: 109130 ccggtttttgttccaagcg 109148
```

☐ >gi|8217498|emb|AL137076.6| Human DNA sequence from clone RP5-893G23 on chromc Contains STSs and GSSs, complete sequence
Length = 89259

Score = 38.2 bits (19), Expect = 9.3
Identities = 19/19 (100%)
Strand = Plus / Plus

```
Query: 111    ctggctgtgtgggaagcag 129
               ||||||||||||||||||
Sbjct: 85099  ctggctgtgtgggaagcag 85117
```

Get selected sequences | Select all | Deselect all

```
Lambda      K       H
    1.37     0.711      1.31

Gapped
Lambda      K       H
    1.37     0.711      1.31
```

Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 2635387
Number of Hits to DB: 5,176,449
Number of extensions: 234481
Number of successful extensions: 3596
Number of sequences better than 10.0: 2
Number of HSP's better than 10.0 without gapping: 2
Number of HSP's gapped: 3596
Number of HSP's successfully gapped: 2
Number of extra gapped extensions for HSPs above 10.0: 3593
Length of query: 259
Length of database: 11,972,756,915
Length adjustment: 21
Effective length of query: 238
Effective length of database: 11,917,413,788
Effective search space: 2836344481544
Effective search space used: 2836344481544
A: 0
X1: 11 (21.8 bits)
X2: 15 (30.0 bits)
X3: 25 (50.0 bits)
S1: 12 (25.0 bits)
S2: 19 (38.2 bits)

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edited by B.N. Fields, D.M. Knipe, P.M. Howley, et al.
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CHAPTER 30

Flaviviridae: The Viruses and Their Replication

Charles M. Rice

Modern studies on flaviviruses began with the discovery, nearly a century ago, that the disease yellow fever (YF) was caused by a filterable agent and transmitted to humans by mosquitoes (205,208,304). The discovery of this viral pathogen eventually led to the derivation of a live-attenuated strain, which has been effectively used for human vaccination for more than 50 years (208,304,318). The *Flaviviridae* (from Latin *flavus*, meaning yellow) was recently established as a separate family (348), distinct from the Togaviridae, and currently includes three genera, the flaviviruses, the pestiviruses (from Latin *pestis*, or plague), and the hepatitis C viruses (from Greek *hepar, hepatos,* or liver) (85). As described in this chapter and in Chapters 31 to 33, these three genera have diverse biological properties and show no serological cross-reactivity, but appear to be similar in terms of virion morphology, genome organization, and presumed RNA replication strategy.

C.M. Rice: Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110-1093.

CLASSIFICATION

Flaviviruses

The *Flavivirus* genus includes more than 68 members separated into groups on the basis of serological relatedness (37) (Table 1; see Chapter 31). More recently, similar relationships have been found by comparison of flavivirus genome sequences (21,171). Most flaviviruses are arthropod-borne, being transmitted to vertebrates by chronically infected mosquito or tick vectors. However, isolates from bats and rodents, without known insect vectors, also have been identified. Arthropod-borne flaviviruses cause significant human and animal disease and are distributed worldwide (206,289) (see also Chapter 31). Clinical symptoms vary and include fever, encephalitis and hemorrhagic fever (see Chapter 31). Entities of major global concern include dengue fever with its associated dengue hemorrhagic fever (DHF) and shock syndrome (DSS) (113,114), Japanese encephalitis (JE) (207), and YF. Tick-borne encephalitis (TBE), Kyasanur Forest disease, West Nile encephalitis (WN), St. Louis encephalitis (SLE), and Murray Valley encephalitis (MVE) are other important agents of regional endemic or epidemic disease (206) (see Chapter 31). Thus far, vaccination is available for YF, using the live-attenuated 17D strain (318), and for TBE and JE using inactivated virus (124).

Pestiviruses

Currently recognized pestiviruses include three serologically related animal pathogens (203) (see Chapter 33). These include the type virus, bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV; also called hog cholera virus), and border disease virus (BDV) of sheep. The border disease group has recently been shown to comprise BVDV-like isolates as well as true BDV strains (16). Pestivirus diseases are widespread and of major economic importance to the livestock industry (203). Transmission occurs by direct or indirect contact as well as by congenital routes. Clinical manifestations vary and include inapparent infections, acute or persistent subclinical infections, fetal death and congenital abnormalities, wasting disease, and an acute fatal illness called mucosal disease (MD) (203). Recently, a new variant of BVDV has been identified that causes severe thrombocytopenia and hemorrhagic syndrome in adult animals (22,63,64,255). Live-

TABLE 1. *Members of the Flaviviridae*

	Group	Type member
Flaviviruses	Tick-borne encephalitis (12[a], T[b])	Central European encephalitis (TBE-W)
		Far Eastern encephalitis (TBE-FE)
	Rio Bravo[c] (6, T)	Rio Bravo
	Japanese encephalitis (10, M)	Japanese encephalitis (JE)
		Kunjin (KUN)
		Murray Valley encephalitis (MVE)
		St. Louis encephalitis (SLE)
		West Nile (WN)
	Tyuleniy (3, T)	Tyuleniy
	Ntaya[c] (5, M)	Ntaya
	Uganda S (4, M)	Uganda S
	Dengue (4, M)	Dengue type 1 (DEN1)
		Dengue type 2 (DEN2)
		Dengue type 3 (DEN3)
		Dengue type 4 (DEN4)
	Modoc (5, U)	Modoc
	Ungrouped[c] (17, M)	Yellow fever (YF)
Pestiviruses	Bovine viral diarrhea	Bovine viral diarrhea (BVDV)
	Classical swine fever	Hog cholera or classical swine fever (CSFV[e])
	Border disease	Border disease (BDV)
Hepatitis C viruses[d]	Hepatitis C	Hepatitis C (HCV)

[a]Number of recognized members in each antigenic group [from Calisher et al. (37)].

[b]Arthropod vectors: T, tick; M, mosquito; U, unidentified or no vector.

[c]Arthropod vectors for some members of these groups have not been identified. The ungrouped flaviviruses include mosquito- and tick-transmitted viruses as well as some with no known vector.

[d]The hepatitis C viruses include a large number of isolates that can be divided into several groups or genotypes on the basis of genetic divergence (36,269,291). An official name for this genus and a standardized nomenclature for different genotypes have not yet been agreed upon.

[e]In the pestivirus literature, HCV has been a common abbreviation for hog cholera virus. More recent publications and this chapter use CSFV to avoid confusion with the human hepatitis C viruses.

attenuated strains and inactivated virus preparations are available for vaccination against CSFV and BVDV (203), but there is need for improved pestivirus vaccines (see Chapter 33).

Hepatitis C Viruses

The hepatitis C viruses (HCV) compose the remaining genus of the Flaviviridae. After the development of diagnostic tests for hepatitis A virus (Chapter 24) and hepatitis B virus (Chapter 86), an additional agent, which could be experimentally transmitted to chimpanzees (4,139,309), became recognized as the major cause of transfusion-acquired hepatitis. The causative agent, previously designated non-A, non-B hepatitis virus and now referred to as HCV, was identified in 1989 (54). Development of diagnostic tests to identify HCV carriers among blood donors (52,162) has already markedly reduced the frequency of posttransfusion hepatitis (3). Humans are the only known natural host for HCV; there is no evidence for vector-mediated transmission. HCV infection is found throughout the world, and the prevalence of anti-HCV antibodies ranges from 0.4% to 2% in most developed countries to more than 14% in Egypt (129) (see Chapter 32). Besides transmission via blood or blood products, or less frequently by sexual and congenital routes, sporadic cases occur that are not associated with known risk factors and account for more than 40% of HCV cases (5,194). Infections are usually chronic (6), and clinical outcomes (138) (see Chapter 32) range from an inapparent carrier state to acute hepatitis, chronic active hepatitis, and cirrhosis, which is strongly associated with the development of hepatocellular carcinoma (HCC) (288). Although alpha interferon has been shown to be useful for the treatment of some patients with chronic HCV infections (65,71) and subunit vaccines show some promise in the chimpanzee model (53), future efforts are needed to develop more effective therapies and vaccines. The considerable diversity observed among different HCV isolates (36,290), the emergence of genetic variants in chronically infected individuals (76,131,151,152, 163,170,227,336,337), and the lack of protective immunity elicited after HCV infection (81,245) present major challenges toward these goals.

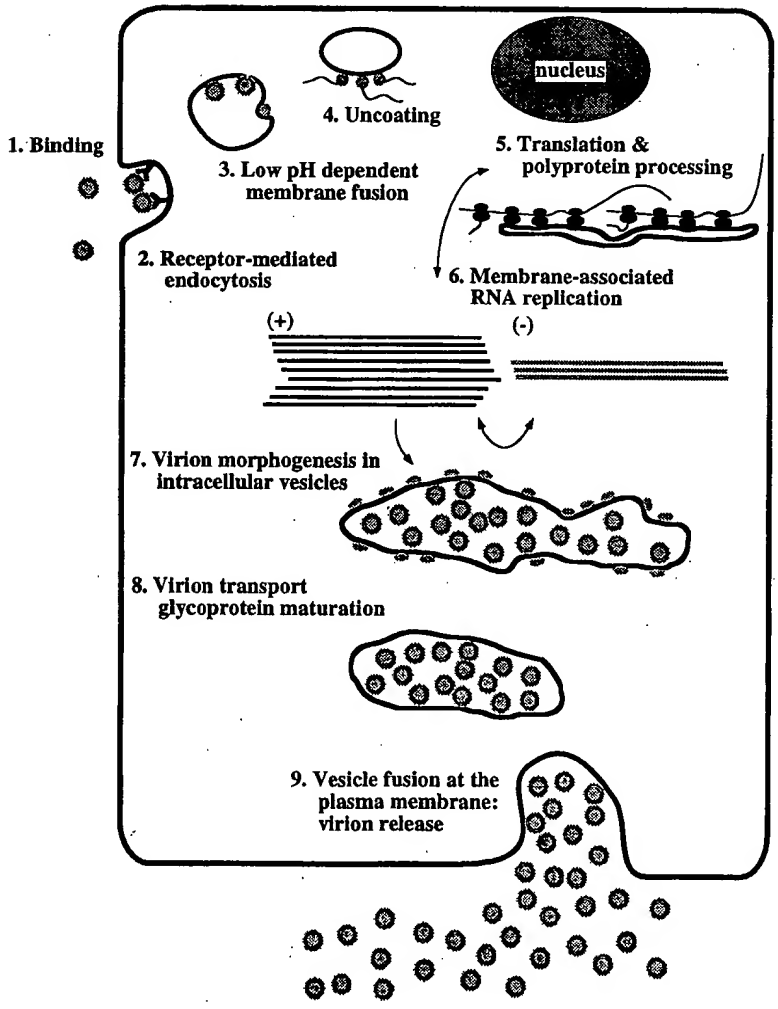

FIG. 1. The flavivirus lifecycle.

FAMILY CHARACTERISTICS AND REPLICATION CYCLE

Common features believed to be shared by the three genera and highlights of the replication cycle are diagrammed in Fig. 1. Our understanding of these steps is far from complete, and the current view is based largely on studies with flaviviruses. Modifications are likely as more information becomes available, particularly for the pestiviruses and HCV. Enveloped virions are composed of a lipid bilayer with two or more species of envelope (E) proteins surrounding a nucleocapsid, which consists of a single-stranded positive-sense genome RNA complexed with multiple copies of a small, basic capsid (C) protein. Binding and uptake are believed to involve receptor-mediated endocytosis, but cellular receptors specific for viral envelope proteins have not been identified. Fusion of the virion envelope with cellular membranes delivers the nucleocapsid to the cytoplasm where translation of the genome RNA occurs. The organization of the genome RNA is similar for all genera. All known viral proteins are produced as part of a long polyprotein of >3,000 amino acids, which is cleaved by a combination of host and viral proteases. The structural proteins are encoded in the *N*-terminal portion of the polyprotein with the nonstructural proteins in the remainder. Sequence motifs characteristic of a serine protease, RNA helicase, and RNA-dependent RNA polymerase are found in similar locations in the polyproteins of all three genera (200). The cleavage products containing these regions are believed to form the enzymatic components of the RNA replicase. RNA replication is cytoplasmic and membrane associated, resistant to actinomycin D, and occurs via synthesis of a full-length negative-strand RNA intermediate. Progeny virions assemble by budding through intracellular membranes into cytoplasmic vesicles. These vesicles follow the host secretory pathway, fuse with the plasma membrane, and release mature virions into the extracellular milieu.

THE FLAVIVIRUSES

Structure and Physical Properties of the Virion

Flavivirus virions are spherical in shape with a diameter of 40 to 60 nm (Fig. 2A) (215). An electron-dense spher-

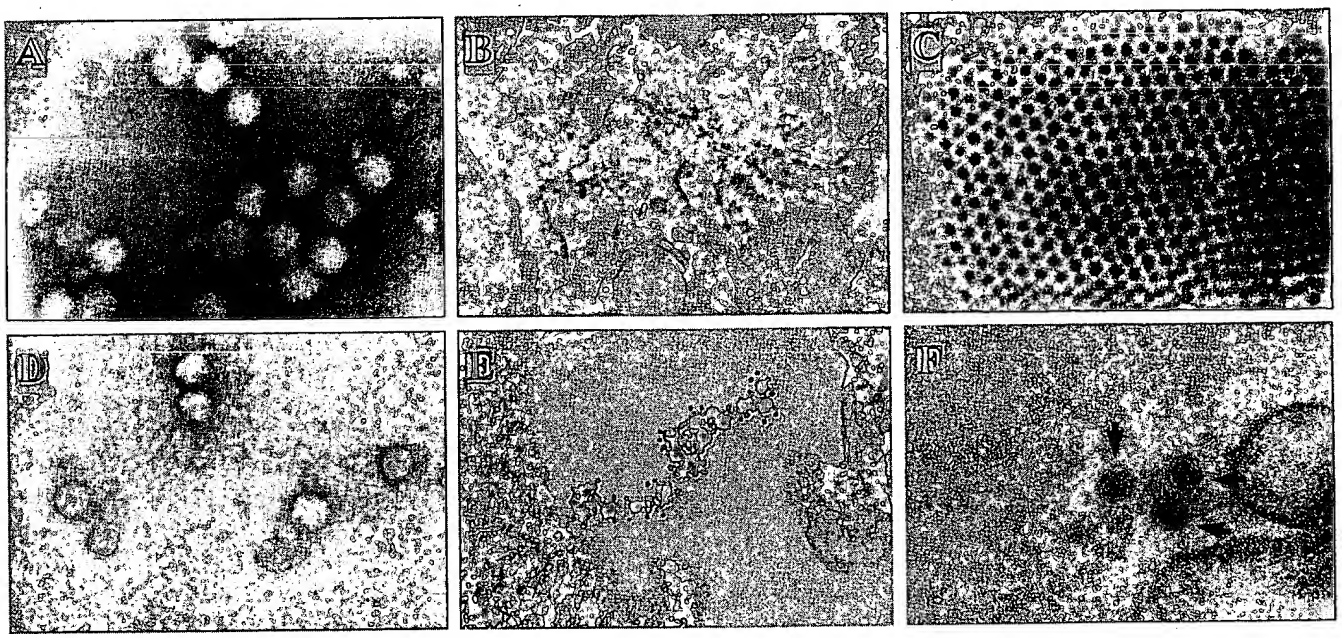

FIG. 2. Electron micrographs of virions and infected cells. **A:** Purified SLE virus negatively stained with ammonium molybdate (215). Surface projections appear as a very thin, indistinct layer (Courtesy of Frederick A. Murphy). **B:** Thin section of a BHK-21 cell at 48 hr postinfection showing SLE virions in the cisternae of the endoplasmic resticulum (349) (Courtesy of Frederick A. Murphy, Sylvia G. Whitfield, and A.K. Harrison). **C:** Paracrystalline array of SLE virus in a *Culex pipiens* mosquito salivary gland cell 25 days after blood meal feeding on an infected suckling mouse (Courtesy of Sylvia G. Whitfield, Frederick A. Murphy, and W. Daniel Sudia). **D:** CSFV virions negatively stained with uranyl acetate (Courtesy of Frank Weiland). **E:** Ultrathin section of STE cells infected with CSFV and immunostained with E0-specific MAb 24/16 and colloidal gold. Bar = 100 nm. From Weiland et al. (334), with permission. **F:** Thin section showing viruslike particles (arrows) in HPBALL cells harvested 14 days after infection with HCV (283). Particles measured approximately 50 nm in diameter (Courtesy of Yokho Shimizu).

ical nucleocapsid ~30 nm in diameter is surrounded by a lipid bilayer. Particles typically have a rather smooth appearance, and regular surface projections are usually not apparent (215), although 7-nm ring-shaped structures have been observed on the surface of DEN virus particles (293). Mature virions sediment between 170 and 210 S, have a buoyant density of 1.19 to 1.23 g/ml, and are composed of 6% RNA, 66% protein, 9% carbohydrate, and 17% lipid (267,324). The envelope proteins—E (envelope) and M (membrane)—are type I membrane proteins embedded in the lipid bilayer by *C*-terminal hydrophobic anchors. Released virions also contain variable amounts of the unprocessed M precursor (prM) (267). Because of the lipid envelope, flaviviruses are readily inactivated by organic solvents and detergents (267). Discrete nucleocapsids, composed of C (capsid) protein and the genomic RNA (120 to 140 S, buoyant density 1.30 to 1.31 g/ml), can be isolated after solubilization of the envelope with nonionic detergents (267). Released extracellular virus particles are morphologically indistinguishable from cell-associated particles found within intracellular vesicles (215). As discussed below, these immature particles contain exclusively unprocessed prM and are less infectious than released virions.

Tropism and Early Events in Flavivirus Infection

Flavivirus replication can be supported by cell cultures derived from many mammalian, avian, and arthropod sources (150). The early events in flavivirus infection are not well characterized. Cellular receptors specific for flavivirus virion glycoproteins have not been identified; however, other cell surface molecules can mediate binding and uptake under specific conditions. For example, in the presence of subneutralizing concentrations of antibody bound to virus, Fc receptors (93,94,124,177,181,182,211–213, 240,275) or C3 complement receptors (38,39) can mediate attachment and uptake. This entry mechanism, termed antibody-dependent enhancement (ADE), may play a role in development of DHF/DSS in sequential infections with different DEN serotypes or in infants with maternal antibody (114,115). After binding, it is generally believed that virions are taken up by receptor-mediated endocytosis (88,146,222), although direct fusion at the plasma membrane also has been observed (119,120). Ultrastructural studies have localized single virions and virion aggregates to clathrin-coated pits on the cell surface, and uptake of virus particles into coated vesicles rapidly follows attachment (88,90). Virions are later found in uncoated prelysosomal vesicles, where an acid-catalyzed membrane fusion is thought to release the nucleocapsid into the cytoplasm (89,90,128). Consistent with this, a conformational change in the viral E protein, which is believed to expose a fusogenic domain (260), occurs at low pH (107,128,154,261). Acid pH can promote fusion of virions with liposomal membranes or at the plasma membrane (89,90,108,154, 253,306), although in the latter case this mode of entry does not lead to productive infection (90,154). The uncoating of nucleocapsids, translation of the incoming genome RNA, and the initiation of RNA replication have not been studied directly.

Genome Structure

The genome of flaviviruses is a single-stranded RNA of approximately 11 kb (28,46,258,347). Genome-length RNAs appear to be the only virus-specific messenger RNA (mRNA) molecules in flavivirus-infected cells. The genomic RNA has a type I cap at its 5′ end (m^7GpppAmp) followed by the conserved dinucleotide sequence AG. Genomic RNAs of mosquito-borne and tick-borne flaviviruses lack a 3′ terminal poly (A) tract and terminate with the conserved dinucleotide CU.

The major portion of the genome RNA consists of a long open reading frame (ORF) of more than 10,000 bases (46). Flanking this ORF are 5′ (95 to 132 bases in length) and 3′ (114 to 624 bases in length) nontranslated regions (NTR) containing conserved RNA elements, which may play as yet undefined roles in RNA replication (Fig. 3). Potential secondary structures have been predicted near the 5′ termini of several flavivirus genomic RNAs with the corresponding structures possible at the 3′ end of minus strand RNA (29,187). For all flaviviruses, secondary structures, conserved in conformation and stability but not in primary sequence, can be predicted for the 3′ terminal 90 bases of the genome RNAs (30,98,110,111,186–188,257,305, 311,339,362). Conserved RNA sequences are also found near the 5′ and 3′ ends of the genome RNAs, but these elements are distinct for mosquito-borne and tick-borne flaviviruses. For the mosquito-borne flaviviruses, two short conserved RNA sequences (called CS1 and CS2; Fig. 3) are located 5′ to the putative 3′ terminal secondary structure (110). CS1 is about 26 nucleotides in length and is located immediately adjacent to the terminal secondary structure. Part of CS1 is complementary to a conserved sequence near the 5′ end of the genome in the region encoding the capsid protein (5′CS). Base-pairing of these or other terminal sequences could lead to cyclization of the viral genome, which may be important for regulating translation, replication, or packaging (29,110,187,305). CS2 is about 24 nucleotides in length and is located 12 to 22 bases 5′ to CS1. This sequence is duplicated in members of the JE and DEN subgroups. As shown in Fig. 3, blocks of conserved RNA sequence and potential cyclization sequences are also found in the genome RNAs of tick-borne flaviviruses (187). Interestingly, some TBE isolates have an internal poly (A) tract in the 3′ NTR (C. Mandl and F.X. Heinz, personal communication), which was earlier thought to represent the 3′ terminus (188). Besides these conserved RNA sequences and structures, short subgroup-specific repeated sequences of unknown function are also observed (29,43,186–188,257).

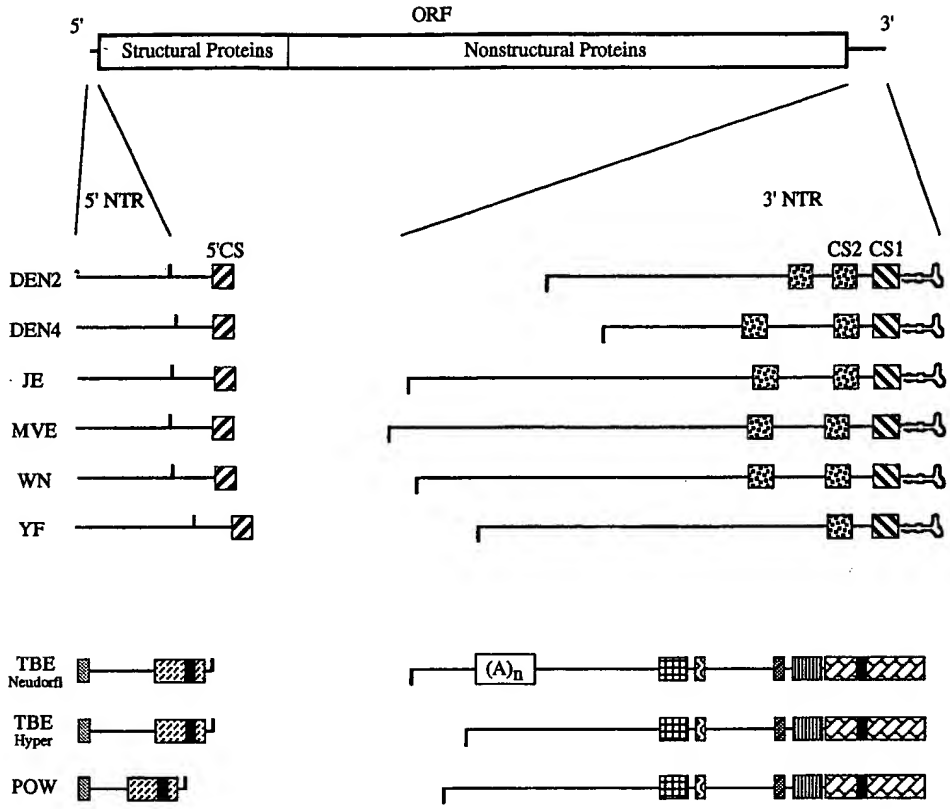

FIG. 3. Conserved RNA elements. A schematic of the flavivirus genome is shown with the 5′ nontranslated region (5′ NTR), the open reading frame (ORF) encoding the structural proteins and nonstructural proteins, and the 3′ nontranslated region (3′ NTR) indicated. Shown below are the 5′ and 3′ regions of several flavivirus genome RNAs highlighting conserved sequence elements (shown as similarly filled boxes) common to mosquito-borne (5′CS, CS1, CS2) or tick-borne viruses. For the tick-borne viruses, the 3′ terminal conserved element can be predicted to form a stable secondary structure similar to that of the mosquito borne viruses. The black boxes at the 5′ and 3′ ends of the tick-borne viruses indicate complementary, potential cyclization sequences. AUG codons or termination codons flanking the ORFs are indicated by ticks above or below the lines, respectively. Adapted from Chambers et al. (46), with unpublished data for TBE (strains Neudorfl and Hyper) and Powassan (POW) kindly provided by Christian Mandl and Franz Heinz.

Translation and Proteolytic Processing

Translation initiation usually occurs at the first AUG in the long ORF but may also occur at a second in-frame AUG located 12 to 14 codons downstream for the mosquito-borne flaviviruses (42). The primary translation product is cleaved cotranslationally and posttranslationally at specific sites by host and viral proteases to produce the virion and replicase components. The structural proteins are encoded in the 5′ quarter of the genome, and the nonstructural (NS) proteins are encoded in the remainder. The order of the cleavage products in the polyprotein is NH_2-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH (46) (Fig. 4). Two small hydrophobic sequences are also cleaved from the polyprotein. These include a segment between the mature virion C protein (virC) and the prM N terminus [the form of the C protein that includes this hydrophobic segment is referred to as anchored C or anchC (7,224)] and a segment preceding the NS4B N terminus [called the "2K" protein (173)].

Structural Region Processing

Numerous *in vivo* and cell-free studies have examined flavivirus polyprotein processing. Translation of the polyprotein occurs largely in association with the rough ER allowing some regions to be translocated into the lumen of the ER, whereas others remain localized on the cytoplasmic side. The current model for polyprotein processing can be summarized as follows (Fig. 4). The nascent C protein contains a C-terminal hydrophobic domain (224) that acts as a signal sequence for translocation of prM into the ER lumen, where core glycosylation of prM occurs. At the C terminus of prM (189,262), adjacent hydrophobic stretches interrupted by a charged residue act as a stop-transfer sequence for prM and as a signal sequence for

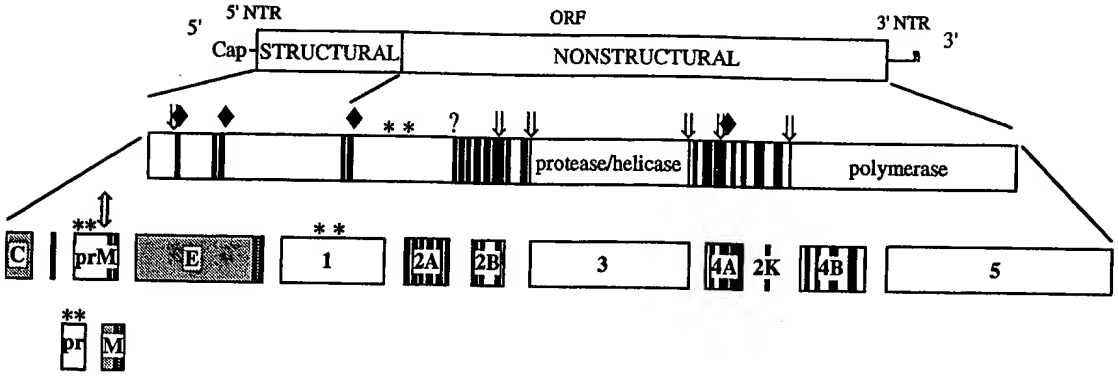

FIG. 4. Translation and processing of the flavivirus polyprotein. At the top is the viral genome with the structural and nonstructural protein coding regions, the 5′ cap, putative 3′ secondary structure, and the 5′ and 3′ NTRs indicated. Boxes below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade. Mature structural proteins are indicated by shaded boxes, and the nonstructural proteins and structural protein precursors by open boxes. Contiguous stretches of uncharged amino acids are shown by black bars. Asterisks denote proteins with *N*-linked glycans but do not necessarily indicate the position or number of sites used. Cleavage sites for host signalase (◆), the viral serine protease (⇓), furin or other Golgi-localized protease (⇓), or unknown proteases (?) are indicated.

translocation of the E protein. Two adjacent hydrophobic sequences at the C terminus of the E protein act similarly for E protein stop-transfer and NS1 translocation. Translocation of NS1 into the lumen of the ER is followed by cleavage at the E/NS1 site and core glycosylation of NS1. Signal peptidase appears to mediate the cleavages generating the N termini of prM, E, and NS1. This view is based on the characteristics of the sequences preceding these cleavage sites, the requirement for microsomal membranes in cell-free translation experiments, and analysis of deletion constructs (78,102,189,262,308,338). The cleavage generating the C terminus of the mature virion C protein is mediated by the virus-encoded NS2B+ NS3 protease complex (called the NS2B-3 protease) (7) and appears to be a prerequisite for efficient signalase-mediated processing at the anchC/prM cleavage site (7,180,356). As discussed below, the prM cleavage is delayed and occurs shortly before virion release. The prM cleavage site sequence, Arg-X-Arg/Lys-Arg (where X is variable), is similar to that used in the maturation cleavages of numerous viral glycoproteins (303), and cleavage may be mediated by the host enzyme furin or an enzyme of similar specificity (112,301).

Nonstructural Region Processing

The protease responsible for cleavage at the NS1/2A site, which is believed to occur in the lumen of a vesicular compartment, has not been identified, but a conserved sequence of eight residues at the C terminus of NS1 (141,235) as well as downstream NS2A sequences (78) appear to be required for efficient cleavage. Most of the cleavages in the remainder of the nonstructural region are mediated in the cytosol by the NS2B-3 protease, which produces the N termini of NS2B, NS3, NS4A, 2K, and NS5 (Fig. 4). Signal peptidase generates the N terminus of NS4B (47,173,243, 295), but this cleavage requires prior processing by the NS2B-3 protease at the 4A/2K site (173). Other than the case just mentioned, there appears to be no obligate order for processing in the nonstructural region (173,174,220, 243). Although the role that these cleavages play in the assembly of functional RNA replication complexes is unknown, they appear to be important because mutations that inactivate the NS2B-3 protease (50) or block cleavage at certain sites (220) are lethal for virus replication.

This genome organization and processing strategy is believed to result in the following topological organization of the cleavage products with respect to the ER membrane (Fig. 5). This model is supported not only by processing

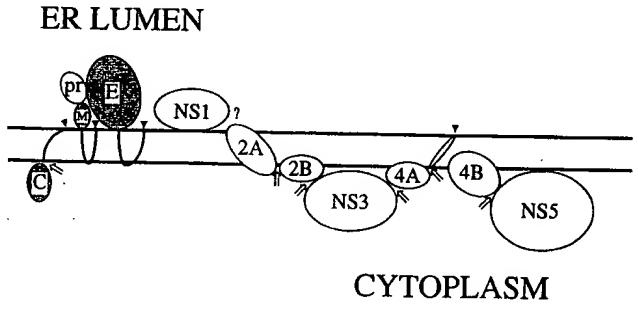

FIG. 5. Model for the membrane topology of the flavivirus proteins. A diagram of the flavivirus polyprotein cleavage products with respect to the ER membrane is shown. The proteins are drawn to scale (areas are proportional to the number of amino acids) and arranged in order (left to right) of their appearance in the polyprotein. Mature structural proteins are shaded and C-terminal membrane spanning segments of M and E are indicated. Also shown are the hydrophobic segment removed from the cleavage sites for host signalase (▼), the viral serine protease (⇓), or an unknown protease responsible for cleavage at the NS1/2A site (?) are indicated.

studies but also by biochemical analyses of membrane fractions isolated from flavivirus-infected cells (44,344). The C protein is localized in the cytoplasmic side and available to form nucleoprotein complexes with genomic RNA molecules. prM and E are type I membrane proteins containing *C*-terminal membrane anchors and *N*-terminal ectodomains localized in the ER lumen. The NS1 glycoprotein, also localized in the ER lumen, does not appear to contain a *C*-terminal hydrophobic anchor. NS2A and NS4B, with their N termini generated by cleavage in the lumen and their C termini generated by the NS2B-3 protease in the cytoplasm, probably span the lipid bilayer at least once. The cleavages generating the remaining polypeptides are all believed to be mediated by the NS2B-3 protease in the cytoplasm. NS2B and NS4A contain hydrophobic regions that are presumably responsible for their membrane association. NS3 and NS5, enzymatic components of the viral RNA replicase (see below), are localized in the cytoplasm and remain loosely associated with membranes perhaps via association with other viral or host components (344).

Features of the Structural Proteins

The C Protein

The virion C protein is a small [predicted molecular mass (M_r) 11 kd], highly basic protein that forms a structural component of the nucleocapsid. Sequence homology among C proteins from different flaviviruses is low, but regions of hydrophobic and hydrophilic amino acids are conserved (185). C protein determinants that participate in RNA and protein interactions important for nucleocapsid assembly have not been defined.

The prM and M Proteins

The prM protein is the glycosylated precursor (M_r 26 kd) of the structural protein M (M_r 8 kd). prM undergoes a delayed cleavage to form M and the *N*-terminal pr segment, which is secreted into the extracellular medium (216). This cleavage occurs shortly before or coincident with virion release because prM and M are found on intracellular and extracellular virions, respectively (Fig. 6). The *N*-terminal pr segment is predominantly hydrophilic and contains one to three *N*-linked glycosylation sites (46) and six conserved cysteine residues, all of which participate in intramolecular disulfide bridges (225). The structural protein M, located in the *C*-terminal portion of prM is present in mature virions and contains a shortened ectodomain (41 amino acids) followed by two potential membrane-spanning domains. Antibodies to prM can mediate protective immunity (153) perhaps by neutralization of released virions that contain some uncleaved prM (see below).

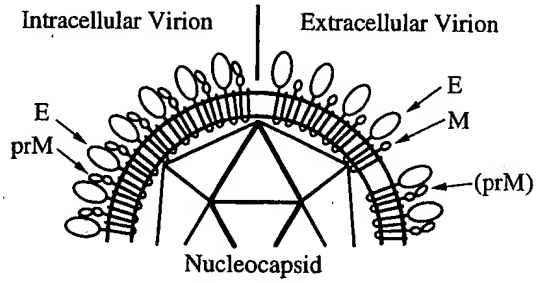

FIG. 6. Envelope proteins of intracellular and extracellular flavivirus virions. Adapted from Chambers et al. (46), with permission.

The E Protein

The E protein is the major envelope protein of the virion. This protein is believed to play key roles in a number of important processes including virion assembly, receptor binding, and membrane fusion, and is the principal target for neutralizing antibodies (see Chapters 31 and ref. 124). Not surprisingly, mutations in this protein can often have dramatic effects on viral pathogenesis (see Chapter 31). Comparison of deduced E protein sequences shows areas of striking homology as well as divergence amongst the flaviviruses (46,126,185,260). All twelve Cys residues in the E ectodomain are strictly conserved and involved in intramolecular disulfide bonds (225). The E protein is glycosylated for some, but not all, flaviviruses, and the role of *N*-linked glycosylation in E function is unclear (46;349a).

A major recent advance has been the determination of a high-resolution structure (256) for a soluble fragment of the TBE E protein ectodomain produced by trypsin digestion of intact virions (127). As detailed in Chapter 31, a wealth of previous information can now be reexamined in the context of this structure. The detergent-solubilized TBE E protein (125) as well as the soluble tryptic fragment used for crytallography is a dimer. The x-ray structure demonstrates that the dimer is a head-to-tail oligomer in the shape of a 170Å rod and predicts that the dimer is anchored in the bilayer at both distal ends. The curvature of the dimer fits with its location on the surface of the 500Å virion and suggests that, consistent with particles visualized by electron microscopy (215), these oligomers do not form long projections or spikes. Two of the three distinct structural entities present in the monomeric unit correspond to previously defined antigenic domains (184). Potential neutralization sites defined by amino acid substitutions present in monoclonal antibody escape mutants are mostly distributed on the surface and can be present in any of the three structural domains (see Chapter 31). The role of the highly conserved sequence from residues 98 through 111, proposed as a fusion sequence (260), is not apparent from the dimer structure. However, the structure of the active fusogenic unit is likely to be an E protein trimer (2) which forms by reorganization of the virion surface upon exposure to acid pH.

Features of the Nonstructural Proteins

The NS1 Protein

The NS1 glycoprotein exists in cell-associated, cell-surface, or extracellular nonvirion forms. NS1 includes 12 strictly conserved cysteine residues, one to three *N*-linked glycosylation sites, and regions of high sequence conservation (46). NS1 is secreted slowly from mammalian cells and is not secreted from mosquito cells (192,241,351). A detergent-stable dimer, which becomes apparent 20 to 40 minutes after synthesis, is the predominant form of both cell-associated and secreted NS1 (169,192,350,351). NS1 expressed by itself can dimerize. Mutagenesis results suggest that the *C*-terminal portion of NS1 is important for dimer stability and secretion (233,246).

The functions of NS1 in virus replication have not been elucidated. Studies with a mutant containing a temperature-sensitive lesion in NS1 suggest a role for this protein early in replication because RNA accumulation is blocked at the nonpermissive temperature (218).

Mutations in NS1 can also affect virulence (239). Natural flavivirus infections elicit antibodies to NS1 with complement-fixing activity, and the secreted form of this protein has been called the soluble complement-fixing (SCF) antigen (267,292). Type-specific, complex-specific, and group-reactive epitopes have been defined for NS1, and some appear to play a role in protective immunity (Chapter 31). Protection may occur via antibody-dependent complement-mediated lysis of virus-infected cells expressing NS1 on the cell surface (276,277).

The NS3 Protein

NS3, the second largest viral protein (predicted M_r ~68 to 70 kd), is highly conserved among flaviviruses (186) and is believed to be an enzymatic component of the RNA replication machinery. Although NS3 does not contain long stretches of hydrophobic amino acids, the protein is membrane associated (344) perhaps via its interaction with the hydrophobic NS2B protein (8,49). Sequence comparisons and biochemical analyses suggest that NS3 is probably at least trifunctional, containing protease, helicase, and RNA triphosphatase activities.

The *N*-terminal one third of NS3 contains the catalytic domain of the NS2B-3 protease as defined by sequence alignment with known serine proteases of the trypsin superfamily (14,15,91), by deletion analysis (50,80,244,340), and by site-directed mutagenesis of the residues in the putative catalytic triad (YF NS3 residues His-53, Asp-77, and Ser-138) (50,247,340,361) or the substrate-binding pocket (242). The sequence surrounding the serine protease nucleophilic serine is conserved among flaviviruses (GXSGXP; YF NS3 residues 136 to 141) (91). The conserved Asp-131 residue and the conserved sequence GLYGNG (residues 151 to 156 for YF NS3) have been hypothesized to form part of the substrate-binding pocket. Besides cleavage at the virC/anchC, 2A/2B, 2B/3, 3/4A, 4A/2K, and 4B/5 sites, the NS2B-3 protease also appears to cleave additional sites in the NS2A (220) and NS3 regions (8). Although polyprotein context and conformation are important determinants of cleavage site specificity (174), cleavage sites usually consist of two basic residues followed by an amino acid with a short side chain [(Arg/Lys/Gln)-(Arg/Lys)↓(Gly/Ser/Ala/Thr)] (46,259). The importance of these conserved residues for cleavage has been examined recently by site-directed mutagenesis (173,174,220).

NS3 also contains significant regions of homology with the DEAD family of RNA helicases (92). This motif is also present in the homologous proteins of other positive-strand RNA viruses and in proteins involved in NTP binding. The sequence alignment of these various proteins generates seven regions of amino acid conservation (located between NS3 residues 191 to 508), including conserved motifs GAGKT, DEAD, and GRXGR, which are postulated to be involved in nucleotide cofactor binding and hydrolysis (92). RNA-stimulated NTPase activity has recently been demonstrated for purified NS3 (333) and for a 50-kd *C*-terminal fragment derived by proteolysis (342). Interestingly, this fragment also contains an RNA triphosphatase activity that is likely to be involved in the formation of the cap structure at the 5′ end of flavivirus genome RNAs (343). Although there is no evidence for cleavage between the protease and helicase domains, autocatalytic processing, possibly at a dibasic site near the end of the NS3 helicase region and preceding the proposed RNA triphosphatase domain (343), has been observed in mammalian but not in mosquito cells (8,248).

The NS5 Protein

The last protein encoded in the long ORF is NS5, the largest (predicted M_r ~103 to 104 kd) and most highly conserved flavivirus protein (186). NS5 is a basic protein, lacking any long hydrophobic stretches, and is believed to be the flavivirus RNA-dependent RNA polymerase. This assumption, although not verified directly, is based on the presence of a highly conserved region, including the sequence motif GDD (YF NS5 residues 666 to 668), which is characteristic of known or putative RNA-dependent RNA polymerases of positive-strand RNA viruses (148,257,258). The *N*-terminal domain of NS5 (between residues 60 and 145) is homologous to a region of methyltransferases implicated in S-adenosylmethionine binding (160). It has been suggested that this domain may be involved in methylation of the 5′ cap structure (160). Although enzymological characterization of the protein is lacking, it seems likely that the NS5 protein is at least bifunctional, possessing both methyltransferase and RNA polymerase activities.

The NS2A, NS2B, NS4A, and NS4B proteins

Less conserved hydrophobic regions of the polyprotein, which are processed to at least four additional nonstructural proteins, are found between the highly conserved NS1, NS3, and NS5 proteins (46,186,258) (Fig. 4). Little is known about their functions in the flavivirus life cycle. In the case of NS2A (predicted M_r ~24 kd), *C*-terminal truncations can inhibit cleavage at the NS1/2A site, but the precise role of NS2A in processing is unclear (78). In the case of YF, at least one additional cleavage site for the NS2B-3 protease occurs in the NS2A region, and a mutation that blocks cleavage at this site is lethal for virus replication (220).

The NS2B protein (predicted M_r ~27 kd) contains a highly charged and conserved central region flanked by hydrophobic segments (79). This protein, together with the NS3 serine protease domain, have been shown to be essential for processing at all of the known structural and nonstructural dibasic sites (7,45,50,80,173,179,243,244, 340,361), and evidence has been obtained for a stable complex between NS2B and NS3 (8,49). Mutagenesis data suggest that the charged central region may participate in the formation of this complex and the active protease (49,79). Besides its function in proteolysis, it has been suggested that the interaction between the hydrophobic NS2B protein and NS3 may be partially responsible for localization of the RNA replication machinery to cellular membranes.

No data are available concerning the function of the hydrophobic NS4A and NS4B proteins. The NS4A cleavage product has been identified only for KUN (296), but incomplete cleavage at the 3/4A site leads to the accumulation of minor amounts of an NS3-4A polyprotein (45, 48,174,179). It is possible that NS3 and NS3-4A have distinct functions in viral replication. The NS4B protein (predicted M_r ~27 kd) spans the ER membrane at least once with multiple membrane-associated segments and cytoplasmic loops (173). NS4B is modified posttranslationally because it appears initially as a 30-kd protein, which is chased to a 28-kd form (243). The nature of this modification is not known.

RNA Replication

After translation of the incoming genomic mRNA, RNA replication begins with synthesis of complementary negative strands, which are then used as templates for production of additional genome-length positive-stranded molecules. These positive strands can then be used for translation of structural and nonstructural polypeptides or synthesis of negative strands or can be encapsidated into virions (28,324,347). In infected vertebrate cells, actinomycin D–resistant synthesis of flavivirus-specific RNA is detectable within 6 hours, and a progressive increase in positive strand genome-length RNA is observed. Positive strand RNA molecules are synthesized from genome-length negative strand templates by a semiconservative mechanism involving replicative intermediates (RIs) and replicative forms (RFs) (56,57). RFs are defined as duplex RNA molecules; RIs contain double-stranded regions as well as nascent single-stranded RNA molecules. Both RFs and RIs can be detected in infected cells (56,57) and as products of *in vitro* RNA polymerase reactions (13,56,103).

The RNase sensitivity of uniformly labeled RIs allows a prediction of the average number of nascent single strands per RI (10), ranging from ~one [KUN (56)] to five [DEN2 (57)]. Ten to 15 minutes are required for completing synthesis of flavivirus genome-length products, which is about tenfold slower than the rate observed for poliovirus (56,57). Interestingly, differences in the number of nascent RNA molecules and the rate of elongation between poliovirus and flaviviruses correlate with the length of their latent periods (57). Synthesis of positive strand RNA versus negative strand RNA must be regulated because a ratio of positive to negative strand RNA of at least 10 to 1 has been observed at the peak of flavivirus RNA synthesis (57). In contrast to alphaviruses, which shut off the synthesis of negative strand templates (see Chapter 27), negative strand synthesis in flavivirus-infected cells continues throughout the replication cycle (57).

Flavivirus-specific proteins, including presumed replicase components NS3 and NS5, sediment with membrane fractions isolated from infected cells (44,344,346,347). Based on the localization of double-stranded RNA (347) and cell-fractionation studies (55,56,103,347), viral RNA synthesis appears to occur principally on the membranes of the perinuclear endoplasmic reticulum. Although highly purified preparations of flavivirus replicase have not been obtained, subcellular membrane fractions enriched in virus-specific RNA-dependent RNA polymerase activity have been used to optimize and study *in vitro* elongation activity (13,55,56,103–105).

Assembly and Release of Particles from Flavivirus-infected Cells

Ultrastructural studies indicate that virion morphogenesis occurs in association with intracellular membranes (215). Electron microscopic studies of flavivirus-infected cells have consistently observed morphologically mature virions first within the lumen of a compartment believed to be the ER (70,121,122,146,155,168,195,221,229,297, 298). In many studies, virions appear to accumulate within disorderly arrays of membrane-bound vesicles (Fig. 2B). Budding intermediates and clearly distinguishable cytoplasmic nucleocapsids have not been observed often, suggesting that the assembly process occurs rapidly. Dramatic proliferation of intracellular membranous structures is a hallmark of flavivirus infection (Fig. 2B), and vesicular transport through the host secretory pathway is believed to be involved in the transport of nascent virions from the ER

to the cell surface where exocytosis occurs. Budding of virions at the plasma membrane has been observed occasionally (122,195,229,297) and does not appear to be a major mechanism for virion formation.

These ultrastructural observations, together with studies on structural protein biosynthesis, oligomer formation, and the properties of intracellular and released virions, suggest the following model for virion assembly and maturation. The cytoplasmic, highly basic C protein presumably interacts with viral genomic RNA to form a nucleocapsid precursor. The orientation of C, prM, and E with respect to the ER membrane would suggest that nucleocapsids acquire an envelope by budding into the ER lumen. Cosynthesis of E and prM is necessary for proper folding of E (157) and these proteins have been shown to be associated as detergent-stable heterodimers (128,341). However, higher order interactions involved in virion assembly have not been defined. Both prM and E are predicted to have only small cytoplasmic domains, but unlike simple transmembrane anchors, their hydrophobic C termini are highly conserved and could be involved in envelope-nucleocapsid interactions. In particular, after disruption of mature virions with some detergents, the M protein can remain associated with the nucleocapsid (267).

Later stages in virion maturation include modification of E (for some viruses) and prM glycans by trimming and terminal addition (46,192,224), implying that virions move through an exocytosis pathway similar to that used for synthesis of host plasma membrane glycoproteins. Although differences in the efficiency of prM cleavage have been noted (216), this cleavage generally distinguishes released virus from intracellular virus particles (280) (Fig. 6). Intracellular M-containing virions have not been detected suggesting that prM cleavage occurs just before release of mature virions. This cleavage can be inhibited by elevating the pH in intracellular compartments (128,254,267,280) and is likely to be catalyzed in post-Golgi vesicles (128,254) by the host enzyme furin or an enzyme with furinlike activity (228). Although inhibition of prM cleavage does not impair virus release, studies on prM-containing particles suggest that this cleavage is required to generate highly infectious virus (106,128,253,280,341). Similar to the alphaviruses (328–330) (Chapter 27), experimental data suggest that the flaviviruses use oligomerization and prM cleavage to regulate the activation of E protein–mediated fusion activity. The current hypothesis is that the function of prM in the prM-E heterodimer is to prevent E from undergoing an acid-catalyzed conformational change during transit of immature virions through an acidic intracellular compartment (106,108,128,254,341). Upon cleavage of prM and release of mature virus, the E-M interaction is destabilized (341). During entry, this allows E to undergo an acid-catalyzed conformational change that promotes fusion in the endosome (106,108,128). The hemagglutination activity exhibited by flaviviruses, which is dependent on low pH, is likely to be the result of activation of this fusogenic activity.

Besides mature virus (170 to 210 S), slowly sedimenting noninfectious particles (70 S), which are also capable of agglutinating red blood cells at acid pH [slowly sedimenting hemagglutinin (SHA) (267)], are released from flavivirus-infected cells. These particles appear as 14-nm doughnutlike structures and are composed of E and M, with variable amounts of prM (267). Recent studies have shown that expression of prM and E is all that is necessary for release of SHA-like particles and that these particles are effective immunogens for eliciting protective immunity (158,159,193,237). Interestingly, expression of longer prM- and E-containing flavivirus polyproteins including the C protein does not lead to release of extracellular particles unless an active NS2B-3 protease is present (180,356) to mediate cleavage at the anchC dibasic site (7).

Effects of Flavivirus Infection on Host Cell Biology

Marked differences in cytopathogenecity are observed depending on the particular flavivirus and host cell type (217,302). In vertebrate cells, dramatic cytopathic and ultrastructural changes can occur, including vacuolation and proliferation of intracellular membranes (215); infection is commonly cytocidal, although some vertebrate cell types do not show these effects and become chronically infected. Even during the peak of virus production, a major inhibition of host macromolecular synthesis is not observed (28,346,347). Arthropod cells in culture may demonstrate cytopathic effects, which are most frequently manifested by cell fusion and syncytium formation (28,302,324). However, infection of mosquito cells is often noncytopathic, and persistent infections can be established (217). Mosquitoes remain chronically infected for life and produce extremely high levels of infectious virus particles in the salivary glands (Fig. 2C).

Generation of Defective Flaviviruses and the Involvement of Host Resistance Genes

Defective-interfering (DI) particles have been valuable tools for the study of RNA virus replication and may play a role in viral pathogenesis in some hosts. For some virus families, strongly interfering DI particles, containing truncated and rearranged genomic RNAs, are easily generated by high-multiplicity passage (see Chapters 5 and 6). These RNAs contain *cis*-acting sequences necessary for replication and packaging but do not encode a complete or functional set of viral proteins. They therefore need a helper virus to supply these functions in *trans*. In the case of flaviviruses, although DI particles have been observed in persistently infected vertebrate cell cultures, strongly interfering DIs are not readily obtained under these conditions or during serial high multiplicity passaging (28). Several potential explanations exist for this observation, including the possibility that, under the conditions tested, most of the virus-spec-

ified components of the RNA replication machinery are required in *cis*. However, at least some components can function in *trans* since complementation at low levels has been demonstrated between temperature-sensitive mutants of JE (74) or SLE (140) are defective in RNA synthesis.

A system in which DIs appear to be readily generated has been studied in some detail (28). Studies that began with the observation of heritable susceptibility to YF virus in mice (274) led to the discovery that a single autosomal-dominant locus can confer resistance to flavivirus infection (28,270,271). Flaviviruses can replicate in such resistant mice, but the spread of infection is slower, with significantly lower peak viremias (10^3- to 10^4-fold) than in congenic susceptible mice. In primary fibroblasts from resistant mice, viral RNA synthesis is reduced, lower titers of infectious virions are released, and a high proportion of DIs can be found even after a single growth cycle. These results indicate that a specific, but as yet unidentified, host gene can dramatically alter flavivirus RNA synthesis.

THE PESTIVIRUSES

Structure and Physical Properties of the Virion

Compared with the flaviviruses, pestiviruses grow poorly in cell culture and are difficult to purify because of their inefficient release from infected cells and association with cellular debris (167). Recent identification of highly permissive cell lines for propagation of CSFV (209,264) has facilitated visualization of virus particles by electron microscopy (203, 334) (Fig. 2D and E) and the characterization of the structural components of the virion (319). Forty- to 60-nm particles are spherical and enveloped and contain an electron-dense inner core with a diameter of ~30 nm (142). Pestivirus virions band at a buoyant density of 1.134 g/ml in sucrose and, like flaviviruses, are inactivated by heat, organic solvents, and detergents (264). Unlike flaviviruses, which are rapidly inactivated by low pH, pestiviruses can survive over a relatively broad pH range (172). The chemical composition of highly purified preparations of pestivirus particles has not been determined, but in addition to the genome RNA and lipid bilayer, four structural proteins are present. These proteins include three envelope glycoproteins [E0, E1, and E2, following the nomenclature proposed by Thiel et al. (319); see Chapter 33] and the capsid protein (C).

Host Range and Early Events

Pestiviruses infect pigs and domestic as well as wild ruminant species (see Chapter 33). In these animal hosts, viral antigens and infectious virus can be detected in a variety of tissue types including epithelial cells at the site of entry, endothelial cells, lymphoreticular cells, and macrophages. In persistently infected animals, BVDV can be detected in most tissues, including peripheral blood mononuclear cells, the gastrointestinal tract, and neurons (see Chapter 33). Primary and continuous cell lines from natural host species are usually permissive for pestivirus replication in cell culture, although considerable differences in replication efficiencies have been noted (142,264). Specific cell surface receptors for pestiviruses have not been identified, and the mechanism of entry and the early events in virus replication have not been extensively studied. One study identified a 50-kd cell surface protein as a candidate receptor using an antiidiotypic antiserum (directed against E2-specific antibodies), which can block BVDV binding to bovine cells (354). Infection of permissive tissue culture cells is usually noncytopathic; however, variants of the ruminant pestiviruses capable of causing cytopathic effects can be isolated from animals with mucosal disease. Based on this cell culture phenotype, pestivirus isolates are referred to as either noncytopathogenic (ncp) or cytopathogenic (cp) biotypes.

Genome Structure

The genome RNAs of prototype strains of BVDV and CSFV are single-stranded RNAs 12.3 to 12.6 kb in length (31,60,67,68,196,210). As discussed below, larger genome RNAs containing duplications and rearrangements have been found for some cpBVDV. In the case of BVDV (cp strain NADL) where the 5′ and 3′ terminal sequences have been determined (31), the long ORF of 11,964 bases is flanked by a 5′ NTR of 385 bases and a 3′ NTR of 229 bases (31,60). Pestivirus genome RNAs do not contain 3′ poly (A) (60,196,209) but appear to terminate with a short poly (C) tract. The 5′ terminus has not been analyzed directly, but it has been suggested that the genome RNAs lack a 5′ cap structure (31,69).

Translation and Proteolytic Processing

As for the flaviviruses, no pestivirus subgenomic RNAs have been detected (209,250,263) and genome-length RNAs are believed to serve as mRNAs for translation of the viral polypeptides. The long pestivirus 5′ NTR contains several short ORFs of unknown function and has been predicted to form a highly structured RNA element that may serve as an internal ribosome entry site (IRES) to initiate cap-independent translation of the long ORF (32,69). Pestivirus RNAs are translated poorly in cell-free systems (61,250) and the current model of pestivirus polyprotein processing comes mainly from analysis of virus-infected cells (1,59, 62,266) and expression of pestivirus polyproteins using the vaccinia or baculovirus systems (236,265,266,352). Most of the cleavage products have been localized in the polyprotein using region-specific antisera (59,300). The proteolytic processing sites in the structural region have been determined by *N*-terminal sequencing (266,299). However, none

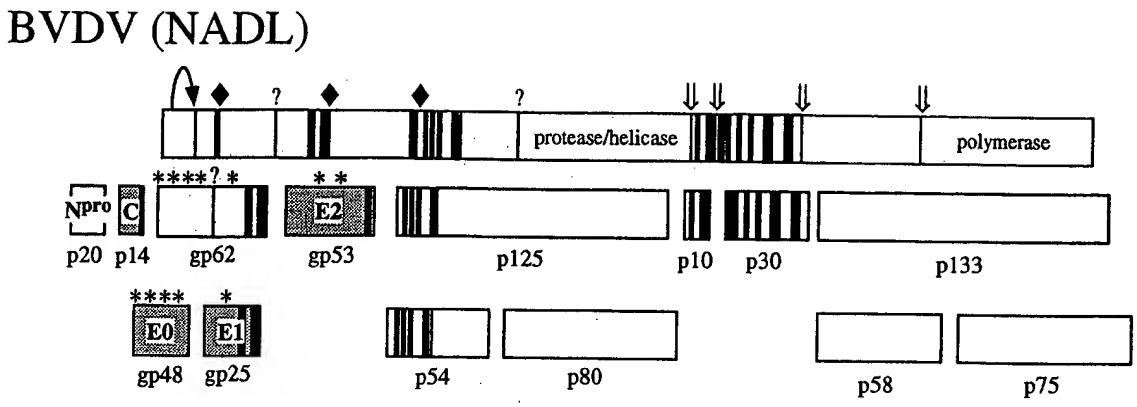

FIG. 7. Processing of the pestivirus polyprotein. Shading and symbols identifying proteolytic cleavages are the same as those described in Fig. 4, except for the proposed autocatalytic cleavage releasing the *N*-terminal N^{pro} nonstructural protein from the pestivirus polyprotein (299,352), which is indicated by the curved arrow. The observed sizes for the proteins (p) and glycoproteins (gp) of the cpBVDV NADL strain (59,62,199) are indicated.

of the cleavage sites in the nonstructural region are known. The order of the cleavage products in the BVDV-NADL polyprotein is NH_2-N^{pro}-C-E0-E1-E2-p125(p54-p80)-p10-p30-p58-p75-COOH (59,62,199) (Fig. 7).

Unlike the flaviruses, the first pestivirus protein encoded in the long ORF is a nonstructural autoprotease responsible for cleavage at the N^{pro}/C site (299,319,352) (Fig. 7). Processing in the pestivirus structural region appears to be mediated by at least two additional proteases. Although some of the cleavages are slightly delayed, host signal peptidase is believed to cleave at the C/E0 and E1/E2 sites and the site generating the C terminus of E2 (266). The E0E1 polyprotein (gp62) is converted slowly to the mature products (266). The protease responsible for this cleavage is unknown. E1 and E2 are believed to be anchored in the lipid bilayer via *C*-terminal membrane segments and some E0 remains associated with the virion via noncovalent interactions that have not been defined (266). Although the existence of a small hydrophobic peptide between E2 and p54 (or p125) has been postulated, no information is available on processing in this region. The pestivirus homologue of the flavivirus NS3 protein is p80, which contains the serine protease domain and is responsible for processing at four downstream nonstructural cleavage sites to generate p10, p30, p58, and p75 (Fig. 7) (353). Differences in p125 processing are observed between pestivirus isolates (16). ncpBVDV isolates do not process p125, whereas BDV isolates are able to cleave p125 inefficiently and produce p80. For CSFV and cpBVDV biotypes, cleavage of p125 is efficient but incomplete so that both p125 and p80 are observed. In the case of the cpBVDV biotypes, the cleavage generating the p80 N terminus is produced via several different mechanisms involving RNA recombinational events (197–199,251,316,317) (see below). Enzymes responsible for cleavage at the p54/p80 site in the CSFV and BDV polyproteins have not been elucidated.

Features of Pestivirus Proteins

As mentioned earlier, the first protein in the pestivirus polyprotein is a nonstructural protein (319), the N^{pro} autoprotease (299,352). This enzyme cleaves at its C terminus at the sequence Cys↓Ser, which is conserved among pestiviruses (299). N^{pro} can also cleave when Ala (352) or Gly (199) is substituted for the Ser residue at the P1′ position. It has been suggested that this protease may be a papainlike cysteine protease (299). N^{pro} is followed by the virion nucleocapsid protein C, a conserved, highly basic 14-kd polypeptide consisting of 21% Lys with a net positive charge of +12. The C terminus of the virion C protein has not been defined, and it is unknown if it retains the hydrophobic segment postulated to serve as the signal sequence initiating translocation of E0 into the ER lumen. The E0 glycoprotein (gp44/48), which contains seven to nine potential *N*-linked glycosylation sites, is heavily glycosylated and present as disulfide-linked homodimers (319). This protein does not contain a potential membrane-spanning domain and is secreted into the culture medium and noncovalently associated with released virus particles (266,334). Interestingly, E0 has recently been shown to possess a ribonuclease activity with a specificity for uridine residues (143,278). Both E1 (gp33) and E2 (gp55) are predicted to be integral membrane proteins and contain two to three and four to six *N*-linked glycosylation sites, respectively (335). E1 and E2 are associated as disulfide-linked heterodimers that form slowly (266); E2 is also present in homodimers (319,335). Although the precise roles of the viral glycoproteins in virus assembly and entry remain to be defined, monoclonal antibodies to E0 (334) or E2 (72,234, 326,335,345) can neutralize virus infectivity, and both antigens can elicit protective immunity (144,265,327).

Much less is known about the pestivirus nonstructural proteins and their roles in virus replication. The p54 pro-

tein has been mapped following E2 and a hydrophobic segment of the polyprotein (59,62). This protein is present as the *N*-terminal portion of p125 and is found as a mature cleavage product only for some cpBVDV strains (16). Near the predicted C terminus of p54, a segment with homology to the zinc finger motif present in some DNA binding proteins has been noted (66). As for the homologous flavivirus NS3 protein, p80 contains an *N*-terminal serine protease domain (14,46,91,353) and motifs characteristic of RNA helicases (92). The p80 protein of BVDV has been purified and shown to possess RNA-stimulated NTPase (313) and RNA helicase (P. Warrener and M. Collett, personal communication) activities. Uncleaved p125 (p54-p80) must be capable of functioning in pestivirus replication because this protein is not processed in cells infected with ncpBVDV strains. The hydrophobic p10 and p30 nonstructural proteins are similar in size, composition, and hydropathic properties to the NS4A and NS4B proteins of flaviviruses and HCV. However, other than a possible role in p80-mediated processing at the p58/p75 cleavage site (353), the functions of these proteins remain undetermined. The remaining two proteins, p58 and p75, are present as mature cleavage products as well as a p133 polyprotein (59,62). p75 contains the GDD motif and is therefore believed to be the pestivirus RNA-dependent RNA polymerase (58,60). Although p58 might correspond to the *N*-terminal portion of the flavivirus NS5 protein, significant homology between these proteins has not been reported. p58 lacks the motifs present in NS5 postulated to be involved in methyltransferase activity (160). Similarly, the *C*-terminal portion of p80 does not contain the proposed RNA triphosphatase domain found in the flavivirus NS3 protein (343). Both of these observations are consistent with the notion that pestivirus genome RNAs lack a 5′ cap structure.

RNA Replication

Detailed analyses of pestivirus RNA replication have not been reported, and RFs and RIs have not been characterized. Accumulation of genome-length intracellular pestivirus RNAs, which comigrate with virion RNA, generally follows the time course of release of infectious virus where maximal virus titers are achieved by 12 to 24 hr postinfection (209,249). As for other positive-strand RNA viruses, pestivirus RNA synthesis is resistant to actinomycin D.

Virion Assembly and Release

Other than the features of the virion structural proteins described above, which have only been elucidated recently, little information is available on the assembly and release of pestiviruses from infected cells. Pestivirus structural proteins are not found on the cell surface (100; F. Weiland, personal communication). Electron microscopic examination of virus-infected cells (18,99) suggests that pestiviruses mature in intracellular vesicles and may be released by exocytosis. A substantial fraction of the infectious virus remains cell associated, and some can be released from infected cells by successive freeze-thaw cycles (166,209).

Pathogenesis of Mucosal Disease and the Generation of Cytopathogenic BVDV via RNA Recombination

Mucosal disease (MD) is the most severe outcome of BVDV infection and is usually fatal (9,33,34). This disease can occur when a fetus is infected in utero with an ncpBVDV strain. If infection with ncpBVDV occurs at 80 to 100 days of gestation, animals may become tolerized to BVDV antigens and remain persistently infected for life. In the rare case of a persistently infected animal exhibiting MD, both cytopathogenic and noncytopathogenic biotypes of BVDV can be isolated. The close serologic relatedness of ncp/cp pairs isolated from an MD-affected animal led to the suggestion that cpBVDV might arise from ncpBVDV by a rare mutational event. Molecular characterization of a number of these ncp/cp pairs has verified this hypothesis and led to the remarkable discovery that some cpBVDV biotypes are generated via RNA recombination (101,197–199,317). In every case studied thus far, these rare events led to the production of p80 (in addition to p125), which is thought to be responsible for cpBVDV cytopathogenicity in cell culture and the pathogenesis of MD in the immunotolerant animal. The sequences of several independent cpBVDV isolates have been determined. Several examples are diagrammed in Fig. 8. In the case of the Osloss and NADL strains, an in-frame insertion of cellular sequences is found just upstream of p80 (197,198). The Osloss insertion is nearly identical to a host ubiquitin monomer; the host sequence found in the NADL strain corresponds to a portion of a bovine mRNA encoding a gene product of unknown function. The CP1 and Pe515 cpBVDV strains contain large duplications encompassing the p80 coding region and insertions of either ubiquitin sequences (198,199) or a duplicated copy of the N^{pro} autoprotease (199), respectively. cpBVDV strain CP9 is actually the first pestivirus-defective interfering RNA described and contains a deletion of the structural p54 coding region, resulting in an in-frame fusion of N^{pro} and p80 (317). For all of these isolates, the insertions/rearrangements led to p80 production either by providing a site for processing by a host protease [ubiquitin carboxyterminal hydrolase for the ubiquitin fusions (316)] or by juxtaposing the *cis*-cleaving N^{pro} viral protease adjacent to the p80 N terminus.

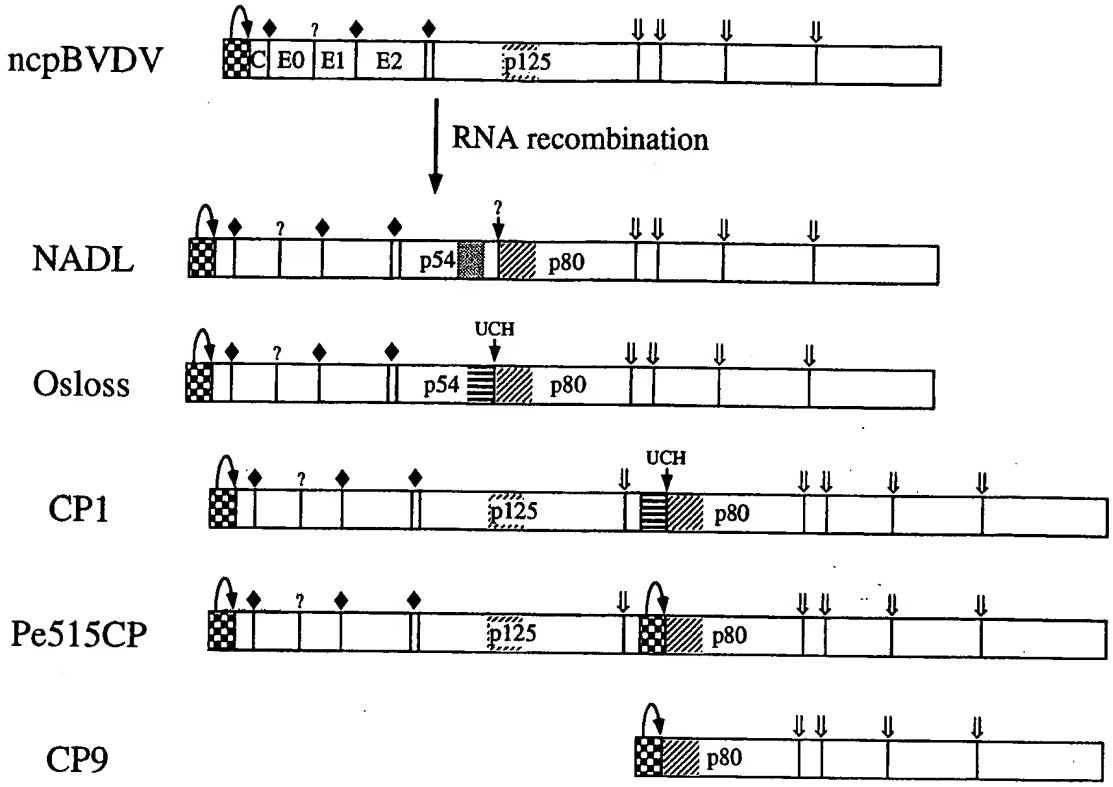

FIG. 8. Genome rearrangements associated with the generation of cpBVDV. The top diagram indicates the polyprotein of a typical ncpBVDV isolate. Below, the polyproteins encoded by five different cpBVDV isolates generated by RNA recombination are shown: NADL (61,196,197); Osloss (197); CP1 (198); Pe515CP (199); and CP9 (317). For all of the cpBVDV isolates, these polyprotein structures allow the production of both p125 and p80. In-frame insertions of host sequences are present in NADL (shaded region), Osloss (striped region), and CP1 (striped region). The enzyme reponsible for p80 production in the NADL strain is unknown, but the inserted ubiquitin sequences in Osloss and CP1 provide sites for processing by host ubiquitin C-terminal hydrolase (UCH) (316). For Pe515CP and the CP9 DI RNA, the N^{pro} autoprotease (checkered box) mediates the cleavage producing the p80 N terminus. The nomenclature and organization of the cleavage products and the symbols for the normal processing enzymes are defined in Figs. 4 and 7. The serine protease domain (hatched region) is also indicated.

THE HEPATITIS C VIRUSES

Structure and Physical Properties of the Virion

Compared with the other two genera, considerably less information is available on the structure and replication of HCV. Besides the relatively recent elucidation of the causative viral agent (54,162), studies have been hampered by the lack of a cell culture system able to support efficient virus replication and the low titers of infectious virus present in serum. The size of infection virus, based on filtration experiments, is 30 to 80 nm (27,123,359). HCV particles isolated from pooled human plasma (310), present in hepatocytes from infected chimpanzees and produced in cell culture (283), have been tentatively visualized by electron microscopy (147a) (Fig. 2F). Initial measurements of the buoyant density of infectious material in sucrose yielded a range of values, with the majority present in a low-density pool of less than 1.1 g/ml (24). Subsequent studies have used RT PCR to detect HCV-specific RNA as an indirect measure of potentially infectious virus present in sera from chronically infected humans or experimentally infected chimpanzees. From these studies, it has become increasingly clear that considerable heterogeneity exists between different clinical samples, and that many factors can affect the behavior of particles containing HCV RNA (135,320). Such factors include association with immunoglobulins (135) or low-density lipoprotein (320,321). In highly infectious acute phase chimpanzee serum, HCV-specific RNA is usually detected in fractions of low buoyant density (1.03 to 1.1 g/ml) (41,135). In other samples, the presence of HCV antibodies and formation of immune complexes correlate with particles of higher density and lower infectivity (135). Treatment of particles

with chloroform, which inactivates infectivity (26,82), or with nonionic detergents, produces RNA-containing particles of higher density (1.17 to 1.25 g/ml), believed to represent HCV nucleocapsids (135,149,201).

There have been many reports of varying levels of negative-sense HCV-specific RNAs in sera and plasma (84). However, it seems unlikely that such RNAs are essential components of infectious particles because some sera with high infectivity can have low or undetectable levels of negative-strand RNA (286). In addition, particles containing negative-sense RNA are not adsorbed to cells, at least as measured by an *in vitro* infectivity assay using a human T-cell line (286). The virion protein composition has not been determined, but putative HCV structural proteins include a basic C protein and two membrane glycoproteins, E1 and E2 (see below). A 26-kd C-specific antigen has been detected in partially purified virus (310).

HCV Replication

Early events in HCV replication are poorly understood. Cellular receptors for the HCV glycoproteins have not been identified. The association of some HCV particles with beta-lipoprotein and immunoglobulins raises the possibility that these host molecules may modulate virus uptake and tissue tropism. Studies examining HCV replication have been largely restricted to human patients or experimentally inoculated chimpanzees. In the chimpanzee model, HCV RNA is detected in the serum as early as 3 days postinoculation and persists through the peak of serum alanine aminotransferase levels (an indicator of liver damage) (287). The onset of viremia is followed by the appearance of indirect hallmarks of HCV infection of the liver. These include the appearance of a cytoplasmic antigen (287) and ultrastructural changes in hepatocytes such as the formation of microtubular aggregates for which HCV previously was referred to as the chloroform-sensitive tubule-forming agent (25). As shown by the appearance of viral antigens (19,136,161,355) and the detection of positive and negative sense RNAs (84,109,118,164,223,281,312,314), hepatocytes appear to be a major site of HCV replication, particularly during acute infection (219). As discussed in Chapter 32, in later stages of HCV infection the appearance of HCV-specific antibodies, the persistence or resolution of viremia and the severity of liver disease vary greatly, both in the chimpanzee model and in human patients. Although some liver damage may occur as a direct consequence of HCV infection and cytopathogenicity, the emerging consensus is that host immune responses, particularly virus-specific cytotoxic T-lymphocytes, may play a more dominant role in mediating cellular damage (see Chapter 32).

It has been speculated that HCV may also replicate in extra hepatic reservoir(s), particularly in chronically infected individuals. In some cases, RT/PCR or *in situ* hybridization has shown an association of HCV RNA with peripheral blood mononuclear cells, including T cells, B cells, and monocytes (20,23,87,109,204,226,332,358,360,363). Such tissue tropism could be relevant to the establishment of chronic infections and might also play a role in the association between HCV infection and certain immunological abnormalities such as mixed cryoglobulinemia (83), glomerulonephritis, and rare non-Hodgkin's B lymphomas (83,147). However, the detection of circulating negative strand RNA in serum, the difficulty in obtaining truly strand-specific RT/PCR (109), and the low numbers of apparently infected cells have made it difficult to obtain unambiguous evidence for replication in these tissues *in vivo*.

Besides providing clues toward understanding *in vivo* tissue tropism, the development of cell culture systems permissive for HCV infection and replication would greatly facilitate future replication studies. Although a cell culture system capable of efficient HCV replication has not been developed, some progress has been made. Consistent with the *in vivo* observations mentioned above, *in vitro* HCV infection and replication have been reported for human hepatocytes (40,145), peripheral blood leukocytes (214), a human B-cell line expressing EBV antigens (17), and a mouse retrovirus-infected human T-cell line (Molt4-Ma) (285). Thus far, only a small fraction of these cells appear infected. However, the *in vitro* infectivity of different HCV inocula using a permissive subclone of the Molt4-Ma T-cell line correlates well with their *in vivo* infectivity in the chimpanzee model (286). This cell line also has been used to begin examining HCV neutralization and the emergence of neutralization escape mutants during chronic infection (284).

Genome Structure

Full-length or nearly full-length genome sequences of numerous HCV isolates have been reported (175,231,269). Given the considerable genetic divergence among isolates, it is clear that several major HCV genotypes are distributed throughout the world. HCV genome RNAs are about 9.4 kb in length, considerably shorter than the genome RNAs of flavivirsues and pestiviruses. The typical 5′ NTR is 341 to 344 bases long and is the most conserved RNA sequence element in the HCV genome. The length of the long ORF varies slightly among isolates, encoding polyproteins of 3,010, 3,011, or 3,033 amino acids. The 3′ NTR shows considerable diversity both in composition and length (28 to 42 bases) and can terminate with either poly (A) or poly (U), depending on the HCV type (51,117,232,322). Conserved secondary structures, possibly important in replication, have been predicted in the 3′ end of the long ORF and the 3′ NTR (116).

Translation and Proteolytic Processing

Several studies have used cell-free translation and transient expression in cell culture to examine the role of the

5′ NTR in translation initiation (86,325,331,357). This highly conserved sequence contains multiple short AUG-initiated ORFs and shows significant homology with the 5′ NTR region of pestiviruses (35,117). A series of stem-loop structures have been proposed on the basis of computer modeling and sensitivity to digestion by different ribonucleases (32,325) (Fig. 9). Although still controversial (331,357), the results from several groups indicate that this element may function as an IRES, allowing efficient translation initiation at the first AUG of the long ORF (86,325,331). Some of the predicted features of the HCV and pestivirus IRES elements are similar to one another (32). It has been proposed that the 5′ terminal hairpin structure and the short ORFs may function to down-regulate translation (357). The ability of this element to function as an IRES suggests that HCV genome RNAs may lack a 5′ cap structure, although this needs to be examined directly.

The organization and processing of the HCV polyprotein (Fig. 10) appears to be most similar to that of the pes-

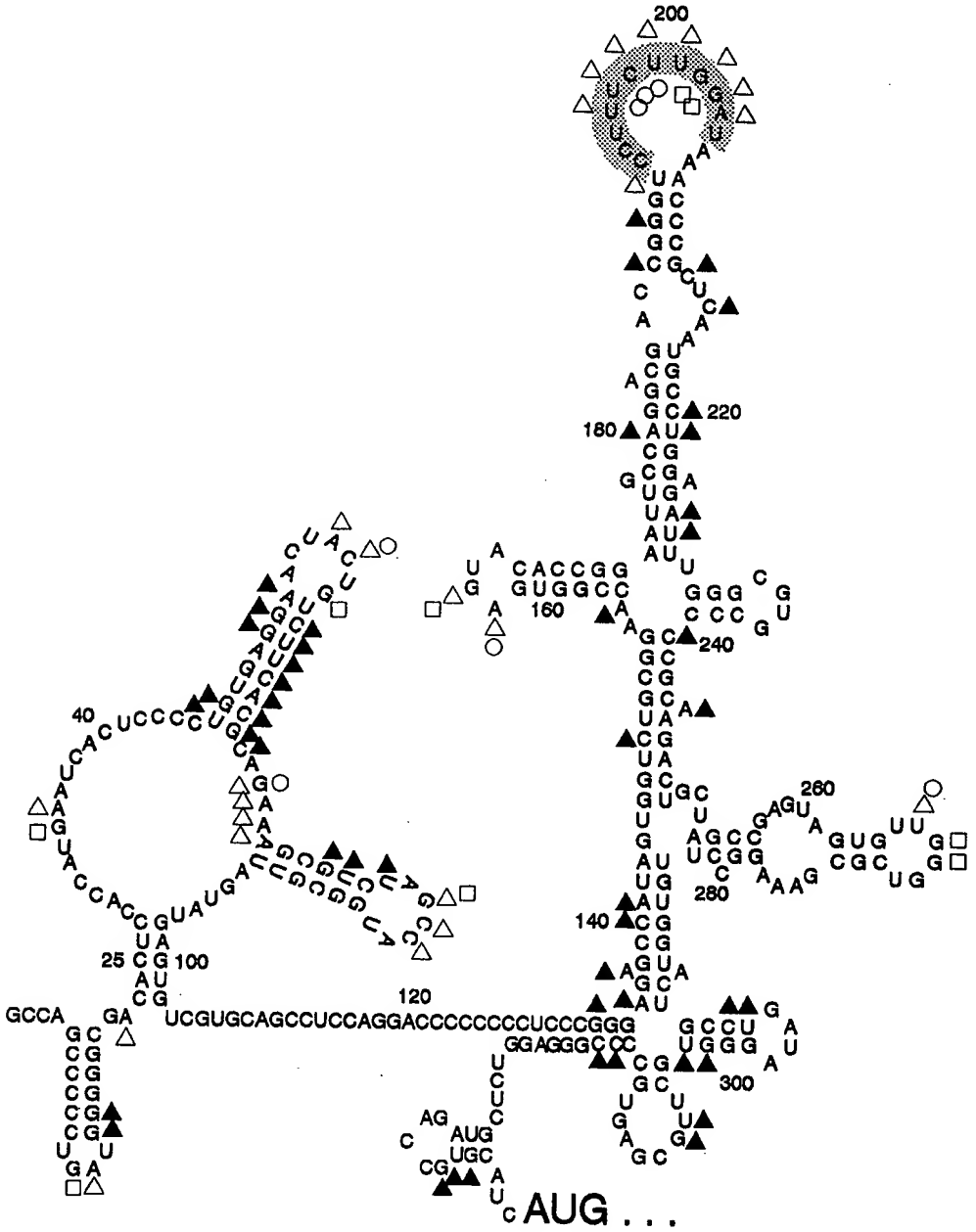

FIG. 9. Predicted secondary structure of the HCV 5′ NTR. Model for the 5′ NTR of HCV (isolate AG94). Experimentally determined sites of cleavage with single-strand or double-strand specific ribonucleases are indicated by symbols adjacent to individual nucleotides. □ = T1, ○ = T2, Δ = S1 (single-strand specific) and ▲ = V1 (double-strand specific). The pyrimidine-rich tract, which is complementary to 18S ribosomal RNA, is indicated by a shaded background. From Brown et al. (32), with permission.

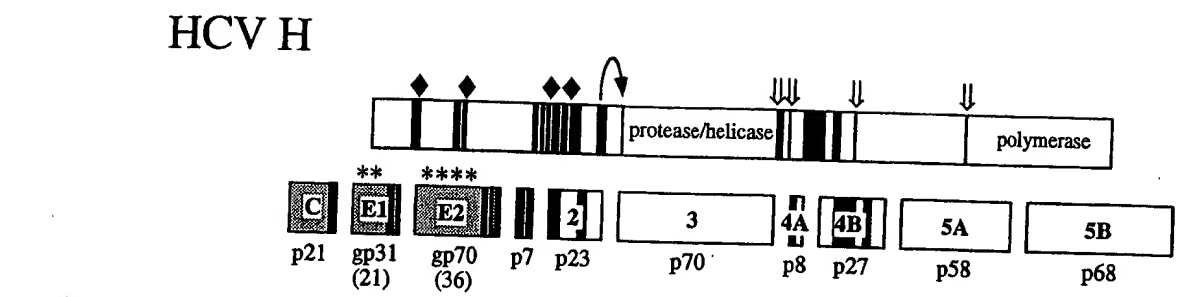

FIG. 10. Organization and processing of the HCV polyprotein. Shading and symbols identifying proteolytic cleavages are the same as described in Fig. 4, except that the curved arrow indicates the autocatalytic cleavage at the 2/3 site catalyzed by the NS2-3 metalloprotease. The observed sizes for HCV-H proteins (p) and glycoproteins (gp) are indicated (97,175). For the glycoproteins the sizes of the endoglycosidase-resistant forms are given in parentheses.

tiviruses. Given the lack of efficient HCV replication in cell culture, our current understanding is based on cell-free transcription/translation and transient expression assays of RNAs derived from HCV cDNA clones (11,75,95–97, 132–134,175,183,202,279,323). At least 10 polypeptides have been identified, and the order of these cleavage products in the HCV-H subtype polyprotein is NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (95–97, 175). As mentioned earlier, the first protein, C, is believed to be the viral capsid protein; E1 and E2 are probable virion envelope glycoproteins; and NS2 through NS5B are putative nonstructural proteins, at least some of which appear to be the functional equivalents of homologous proteins encoded by flaviviruses and pestiviruses. It should be noted that E2 has been referred to as E2/NS1 or NS1 based on possible analogy to the flavivirus NS1 protein. E2 is now the preferred nomenclature because accumulating data suggest that this protein is likely a virion envelope glycoprotein. Whether p7 is a virion component or nonstructural protein is not known.

For one isolate, HCV-H, *N*-terminal sequences have been determined for all of the known cleavage products, allowing precise localization of the processing sites (95,96,175). Limited data for other isolates (75,252) and more divergent subtypes (132,137,202,238), suggest that overall processing schemes are similar. Based on the characteristics of sequences preceding cleavage sites and the results of cell-free translation assays, in which processing is dependent on the addition of microsomal membranes, host signal peptidase in the ER lumen appears to catalyze cleavages in the structural-NS2 region (C/E1, E1/E2, E2/p7, and p7/NS2) (96,132,175,202). Some of these processing reactions are not cotranslational, and glycosylated intermediates are readily detected, as in the case of the pestivirus structural protein region. E2-NS2 appears to be a discrete but short-lived precursor that is cleaved to produce E2, E2-p7, and NS2 (73,175). For some HCV strains, processing at the E2/p7 site is inefficient, leading to the production of a reasonably stable E2-p7 species (73,175). Other cleavages in the polyprotein appear to be mediated by two overlapping HCV-encoded proteases that have different catalytic mechanisms and cleavage site specificities (95,96, 133,137). A putative Zn^{2+}-dependent metalloprotease that encompasses the NS2 region and the *N*-terminal one third of the NS3 protein mediates *cis* cleavage at the two thirds site (96,133). The second viral protease is located in the *N*-terminal one third of the NS3 protein, and as for the flaviviruses and pestiviruses, this serine protease domain is required for several downstream cleavages in the NS region (3/4A, 4A/4B, 4B/5A, and 5A/5B) (11,75,95,133,183, 323). Unlike the flavivirus NS2B-3 protease, the upstream NS2 region is not required for the HCV NS3 serine protease activity (11,75,95).

Features of Hepatitis C Virus Proteins

The C protein is the most highly conserved HCV protein. This basic protein is believed to associate with genome RNA to form the nucleocapsid. As mentioned earlier, a 26-kd C-specific antigen has been detected in HCV nucleocapsids partially purified from pooled human plasma (310). The size of this antigen is substantially larger than the 21-kd product observed in cell-free translation and transient expression systems, which suggests that an alternative or modified form of the C protein may be incorporated into mature virions. As predicted by the signalase-mediated processing at the C/E1 site, the primary C-specific cleavage product may contain a *C*-terminal hydrophobic anchor. However, recent evidence suggests that a second signalase cleavage site may be located approximately 18 residues upstream from the C/E1 site (272). Although this shortened version of C lacks the *C*-terminal hydrophobic segment, it was still found to be associated with the ER membrane. This study also showed that the basic *N*-terminal portion of C possesses RNA binding activity *in vitro*; however, specificity for HCV RNA was not demonstrated (272). There also have been reports of al-

ternative forms of the C protein observed in cell-free translation (178) and vaccinia transient expression (279) assays. Some of these alternative forms, particularly those that are no longer membrane associated, may have other functions in HCV replication. For instance, a recent study found that prolonged expression of the HCV C protein in human hepatoma cells resulted in a shift from cytoplasmic to nuclear localization, which correlated with a suppression of hepatitis B virus (HBV) transcription, assembly, and release (282). This may be related to the observation that HCV superinfection of patients infected with HBV can sometimes lead to suppression or termination of the HBV carrier state (282) and suggests that this protein could also play a role in modulating host gene expression in HCV-infected cells.

The E1 and E2 proteins are heavily modified by *N*-linked glycosylation and are believed to be type I transmembrane glycoproteins with *N*-terminal ectodomains and *C*-terminal hydrophobic anchors. In the case of the E2 protein expressed alone, deletion of the *C*-terminal hydrophobic region results in secretion of the ectodomain (294), as opposed to ER retention for the intact protein (73,252). Potential *N*-linked sites and Cys residues of both proteins are highly conserved among HCV isolates. After synthesis and core glycosylation, most of E1 and E2 fold and associate to form nonionic detergent-stable heterodimers slowly (73,165,252). Most E1E2 heterodimers are stabilized by noncovalent interactions, although a small fraction of these proteins is present in heterogeneous disulfide-linked aggregates (73,97), which most likely represent mis-folded complexes (73). Such aggregates may be artifacts of the high-level expression systems typically used for these studies. As analyzed in transient expression assays, E1 and E2 localize predominantly to the ER network and are not found at the cell surface, which is consistent with the observed lack of complex *N*-linked glycans (73, 252,279,294). The E1E2 heterodimer may represent the basic subunit of the virion envelope, but analysis of highly purified infectious virus and further biological characterization of glycoprotein function are needed to verify this possibility. Purified E1E2 oligomers can be used to elicit protective immunity against challenge with low doses of homologous virus (53), but it is not yet known if protection is mediated by neutralizing antibody or other immune mechanisms. The p7 protein has been only recently identified (175), and, as mentioned above, inefficient cleavage at the E2/p7 site of the HCV-H subtype leads to production of two reasonably stable species: E2 and E2-p7. Further studies are needed to determine if E2 and E2-p7 have distinct, perhaps strain-specific roles in virus replication and whether p7 is a nonstructural protein or a virion component or both.

Aside from its hydrophobic character, no significant sequence homology exists between the HCV NS2 protein and the analogous flavivirus or pestivirus proteins. Site-directed mutagenesis has shown that two residues in the NS2 region, His-952 and Cys-993, are critical for cleavage at the 2/3 site (96,133), which occurs at Leu↓Ala (residues 1026/1027) (96,137). These data, together with the results of deletion analyses, suggest that the NS2 region and the *N*-terminal one third of NS3 form an autoprotease responsible for *cis* cleavage at the two thirds site. This activity appears to require Zn^{2+} and can occur in the absence of microsomal membranes (96,133).

The 70-kd NS3 protein appears to be the functional homologue of the flavivirus NS3 and the pestivirus p125/p80 proteins. The serine protease domain in the *N*-terminal portion is required for cleavage at the four downstream sites. The residues flanking these cleavage sites are highly conserved and conform to the sequence Cys (Thr)↓Ser (Ala) (75,95,238). As shown by site-directed mutagenesis (156) and molecular modeling (238) the P1 Cys (or Thr) residue appears to be an important determinant for efficient cleavage. An acidic residue (Asp or Glu) is found six residues upstream from each of the cleavage sites (95). However, at least for cleavage at the 4A/4B site, an acidic residue at this position is not required for efficient processing (156). Thus, the cleavage site specificity of the HCV serine protease is distinct from that of the flavivirus NS2B-3 protease. The remainder of the HCV NS3 protein consists of a putative RNA helicase domain that has been partially purified and shown to have RNA-stimulated NTPase activity (307). As with the pestivirus p125/p80 protein, HCV NS3 lacks the proposed RNA triphosphatase motifs found in the *C*-terminal portion of the flavivirus NS3 protein.

Cleavage at the 3/4A site by the NS3 serine protease is believed to occur primarily in *cis* (12,323), and accumulating evidence suggests that the NS4A protein somehow facilitates cleavage at this site as well as at other NS3 serine protease-dependent cleavage sites (12,77,176). The *N*-terminal half of the 54-residue NS4A protein is hydrophobic, followed by a highly charged region. The mechanism by which NS4A facilitates NS3 serine protease-dependent cleavages is not known, but presumably involves interactions of NS4A with the protease and/or the substrate. As for the other genera, there is a paucity of information regarding the functions of the remaining nonstructural proteins. NS4B is rather hydrophobic. NS5A and NS5B are hydrophilic, and the latter protein contains the GDD motif and is thus presumed to be the RNA-dependent RNA polymerase. All of these putative nonstructural proteins cosediment with membranes when expressed in surrogate systems (134) and are likely associated with host components to form complexes involved in RNA replication. Although amino acid sequences in the nonstructural regions of the three genera are highly divergent, the hydrophobicity profiles, the organization of conserved motifs, and the location of processing sites are very similar (particularly for HCV and the pestiviruses), suggesting that their replication complexes may have similar architecture and function.

TABLE 2. *Common and distinct features of flaviviruses, pestiviruses, and hepatitis C viruses*

Genus	Genome size	Genome features	Translation strategy	Virion proteins	Secreted glycoprotein?
Flavivirus	~11 kb	5' cap; short 5' NTR; ~3,400 aa ORF; long 3' NTR	Cap-dependent	C: basic, poorly conserved M & E: transmembrane glycoproteins; prM maturation cleavage	NS1
Pestivirus	~12.5 kb	Long 5' NTR; ~3,000 aa ORF; short 3' NTR	IRES?	C: basic, highly conserved. E1 & E2: transmembrane glycoproteins. E0: virion associated	E0: secreted and virion associated; ribonuclease activity
Hepatitis C virus	~9.4 kb	Long 5' NTR; ~4,000 aa ORF; short 3' NTR	IRES?	C: basic, highly conserved. E1 & E2 (and E2-p7?): transmembrane glycoproteins	None identified

Virion Assembly and Release

This process has not been examined directly, but the lack of complex glycans, the ER localization of expressed HCV glycoproteins (73,252), and the absence of these proteins on the cell surface (73,294) suggest that initial virion morphogenesis may occur by budding into intracellular vesicles. Thus far, efficient particle formation and release has not been observed in transient expression assays, suggesting that essential viral or host factors are missing or blocked. As for the pestiviruses, HCV virion formation and release may be inefficient, with a substantial fraction of the virus remaining cell associated. A recent study indicates that extracellular HCV particles partially purified from human plasma do contain complex *N*-linked glycans, although these carbohydrate moieties were not shown to be specifically associated with E1 or E2 (273). Complex glycans associated with glycoproteins on released virions would suggest transit through the *trans* Golgi and movement of virions through the host secretory pathway. If this suggestion is correct, intracellular sequestration of HCV glycoproteins and virion formation could play a role in the establishment and maintenance of chronic infections by minimizing immune surveillance and preventing lysis of virus-infected cells via antibody and complement.

Genetic Variability

As for all positive-strand RNA viruses, the RNA-dependent RNA polymerase of HCV is believed to lack a 3′-5′ exonuclease proofreading activity for necessary removal of misincorporated bases. Replication is therefore error-prone, leading to a quasispecies virus population consisting of a large number of variants (190,191). This variability is apparent at multiple levels. First, in a chronically infected individual, changes in the virus population occur over time (227,230), and these changes may have important consequences for disease. A particularly interesting example is the *N*-terminal 30 residues of the E2 glycoprotein, which exhibits a much higher degree of variability than the rest of the polyprotein (130,131,336). There is accumulating evidence that this hypervariable region, perhaps analogous to the V3 domain of HIV-1 gp120, may be under immune selection by circulating antiviral antibodies (152,315,337). In this model, antibodies directed against this portion of E2 may contribute to virus neutralization and thus drive the selection of variants with substitutions that escape neutralization. This plasticity suggests that the E2 hypervariable region is not essential for other functions of the protein, such as virion attachment, penetration, or assembly.

Common nonstructural proteins/functions			Distinct nonstructural proteins/functions			Viral biology
Serine protease	NTPase/ RNA helicase	RNA dependent RNA polymerase GDD motif	Additional viral proteases	RNA triphosphatase motif?	Methyl transferase motif?	
NS2B & NS3 protease complex	NS3	NS5 (100 kd)	None identified	Yes, *C*-terminal domain of NS3	Yes, *N*-terminal domain of NS5	Usually vectored by mosquitoes or ticks; acute limited infections in vertebrate hosts; usually cytopathic in vertebrate cell cultures
p80/p125 possible requirment for p10?	p80/p125	p75	N^{pro} leader protease	No	No	No known insect vector; chronic infections can be established under some conditions; both cytopathic and noncytopathic isolates
NS3 or NS3 & NS4A protease complex	NS3	NS5B (68 kd)	NS2-3 Zn^{2+}-dependent autoprotease	No	No	No known insect vector; chronic infections common

Genetic variability also may contribute to the spectrum of different responses observed after treatment of chronically infected patients with alpha interferon. Diminished serum alanine aminotransferase levels and improved liver histology, which is sometimes correlated with a decrease in the level of circulating HCV RNA, is seen in only 40% of those treated (100) (see Chapter 32). After treatment, approximately 70% of the responders relapse. In some cases, after a transient loss of circulating viral RNA, renewed viremia is observed even during the course of treatment. Although this might suggest the existence or generation of interferon-resistant HCV genotypes or variants, further work is needed to determine the relative contributions of virus genotype and host-specific differences in immune responsiveness.

Finally, sequence comparisons of different HCV isolates around the world have begun to uncover enormous genetic diversity (see Chapter 32). Because of the lack of serological assays such as cross-neutralization tests, HCV types, subtypes, and isolates are currently being grouped on the basis of nucleotide or amino acid sequence similarity (36, 231,291). Amino acid sequence similarity between the most divergent types can be as little as 50%, depending on the protein being compared. This diversity is likely to have important biological implications, particularly for diagnostics, vaccine design, and therapy.

Association of Hepatitis C Virus with HCC

A significant fraction of chronically infected patients slowly progress from chronic active hepatitis to cirrhosis and then to HCC (283,288). The mean onset for development of primary HCC has been estimated to be 20 to 30 years (268,349). Studies have identified both positive- and negative-strand HCV RNA in tumorous tissue from some, but not all patients. The slow onset and the apparent requirement for preexisting cirrhosis suggest that HCV may not directly cause HCC but rather predisposes the organ to carcinogenic events. However, these observations do not exclude the possibility that expression of particular HCV gene products in chonically infected cells might predispose them to carcinogenesis.

SUMMARY AND QUESTIONS

As mentioned at the beginning of this chapter, the flaviviruses, pestiviruses, and hepatitis C viruses have a number of common features, but also many differences. These are summarized in Table 2. It is now clear, in terms of translational strategy and proteolytic processing schemes, that the pestiviruses and hepatitis C viruses are more closely

related. These two genera also lack arthropod vectors and can establish persistent infections in their respective vertebrate hosts. Although a great deal of progress has been made in our understanding of genome structures, polyprotein processing, and some viral polypeptide functions, large gaps in our knowledge exist for every step in the complex life cycles of these viruses. A few of the many challenging questions that remain include the following: What host cell surface molecules mediate virus binding and uptake? What *cis* RNA elements are responsible for genome translation, replication, and packaging, and how are these elements recognized? What is the importance of proteolytic processing in assembly of functional RNA replication complexes and what is their composition, both in terms of viral and host components? How and where does virion morphogenesis occur and what host secretory pathway is responsible for virion release? What mechanisms are involved in the establishment and maintenance of chronic infections by hepatitis C viruses? Why does production of p80 by cp-BVDV correlate with cytopathogenecity and fatal mucosal disease? Is there a direct link between the expression of HCV-specific gene products and the development of HCC? Answers to these and other pressing questions should provide important information for the development of effective immunization strategies and therapies to control diseases caused by these diverse and important pathogens.

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